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From Poles to Equator: Functional Analysis of DdAurora during Mitosis and Cytokinesis in *Dictyostelium discoideum*

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From Poles to Equator: Functional Analysis of DdAurora during Mitosis and Cytokinesis in *Dictyostelium discoideum*

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Hui Li, B.S.

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Dedication

To my parents, Junhua and Eric

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From Poles to Equator: Functional Analysis of DdAurora during Mitosis and Cytokinesis in *Dictyostelium discoideum*

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The Aurora kinases are highly conserved serine/threonine kinases that play essential roles throughout mitosis. In metazoans, these functions are mediated by Aurora A and B at the spindle poles and the equatorial region respectively. I show here that *Dictyostelium* contains a single Aurora kinase, DdAurora that displays characteristics of both Aurora A and B. Like Aurora A, DdAurora has an extended N-terminal domain with an A-box and localizes to the spindle poles during early mitosis. Like Aurora B, DdAurora localizes to centromeres in metaphase, the central spindle during anaphase and the cleavage furrow at the end of cytokinesis. In addition to these known features of Aurora A and B, I found that DdAurora remains associated with centromeres during anaphase and telophase which has not been shown in any other organisms.

INCENP is known to be an important binding partner of Aurora B. In *Dictyostelium* the conserved C-terminal IN-box domain of DdINCENP is essential for its interaction with DdAurora and for the localization of DdAurora to the central spindle. In contrast, the centromeric and spindle pole localization of DdAurora does not require an interaction with DdINCENP. Surprisingly, a truncated DdINCENP protein lacking the IN-box domain can still localize on centromeres and the central spindle even though it does not bind to DdAurora.

I also found that the localization of DdAurora to the central spindle requires Kif12, a protein similar to mitotic kinesin like protein 2 (MKLP2). However, this requirement is suppressed by the overexpression of GFP-DdINCENP. GFP-DdINCENP can localize to the central spindle in the absence of Kif12 and it probably recruits DdAurora to the same location through their strong interaction. Finally, I demonstrated that Myosin II heavy chain is important for the proper localization of the DdAurora/DdINCENP complex to the cleavage furrow during late cytokinesis.

With the exception of DdINCENP, no other binding partner or substrate of DdAurora has been identified in *Dictyostelium*. By performing large-scale immunoprecipitation in wild-type cells, I identified several potential binding partners/substrates of DdAurora, including topoisomerase B and HspA. Future research on these proteins may help to elucidate DdAurora function in different stages of M phase.

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Chapter 1 Introduction

1.1 AURORA KINASES – A FAMILY OF KINASES ESSENTIAL FOR MITOSIS.

Mitosis is the process by which a cell separates its duplicated genome into two identical halves. It is generally followed immediately by cytokinesis which divides the cytoplasm and cell membrane. The nuclear division stage (mitosis) and cytoplasmic division stage (cytokinesis) together define the mitotic (M) phase of the cell cycle. The M phase is a complex process and requires coordination of many critical events, such as centrosome maturation and separation, chromosome condensation and segregation, spindle assembly and elongation, cleavage furrow formation and contraction. How all these events are coordinated for successful cell division remains unclear.

The Aurora kinase family is a group of conserved serine/threonine kinases that have essential functions for multiple stages of mitosis and cytokinesis. The *Aurora* gene was identified in *Drosophila melanogaster* in a search for genes that regulate the structure and function of the mitotic spindle (Glover et al., 1995). *Aurora* mutants display closely paired centrosomes at inappropriate mitotic stages and develop interconnected spindles in which the poles are shared, suggesting that the protein kinase is essential for centrosome separation. Ipl1 — the single essential Aurora kinase of *Saccharomyces cerevisiae* was identified independently in a genetic screen for mutants that were defective in chromosome segregation (Chan and Botstein, 1993). Subsequently, Aurora kinase homologs in different organisms have received tremendous attention. The Aurora kinase family has now grown to include three related kinases in mammals and plants, two

in flies, frogs and worms, and one in yeast (Adams et al., 2001a). Recent studies into their physiological functions, as well as their substrates, have helped to elucidate the roles of these enzymes during M phase. Based on their function in different stages of mitosis, they have been divided into three subfamilies – Aurora A, B and C (Adams et al., 2001a). The original *Drosophila* Aurora is now classified as an Aurora A kinase, which regulates centrosome maturation and segregation in general. Ipl1 is classified as an Aurora B kinase, which is essential for chromosome condensation, segregation and cytokinesis. Aurora C kinases are only found in mammalian cells and are specifically expressed at high level in testis and involved in meiosis (Kimura et al., 1999). The function of Aurora C is most similar to that of Aurora B (Li et al., 2004). Although Aurora kinases from different subfamilies show a high degree of sequence homology, their localization and function during the cell cycle are quite distinct.

1.1.1 Aurora A – the Polar Aurora

Aurora A has been shown to associate with centrosomes and the adjacent spindle microtubules and is essential for centrosome maturation and separation in various organisms, such as *C. elegans* (Hannak et al., 2001; Schumacher et al., 1998a) and *Drosophila* (Berdnik and Knoblich, 2002; Glover et al., 1995).

Centrosome maturation is a process by which separated centrosomes recruit numerous proteins to become fully functional. In the absence of Aurora A, recruitment of several components of the pericentriolar material – including γ-tubulin – to the centrosome is deficient, and the microtubule mass of spindle is decreased by about 60% (Berdnik and Knoblich, 2002; Hannak et al., 2001). The discovery of an interaction

between Aurora A and transforming acidic coiled-coil (TACC) proteins may be able to explain how Aurora A regulates centrosome maturation (Giet et al., 2002). Phosphorylation of TACC by Aurora A could facilitate recruitment of TACC to the centrosome in early mitosis, leading to the recruitment of Msps/XMAP215, a microtubule-associated protein that promotes the growth of microtubules.

Centrosome separation is a process required for the initial formation of the mitotic spindle. In *C. elegans* embryos lacking Aurora A (AIR-1), centrosome asters can separate but remain associated after nuclear-envelope breakdown (Hannak et al., 2001). This result suggests that Aurora A is not necessary for the initiation of centrosome separation, but is required to maintain centrosome separation during spindle assembly. In HeLa cells, micro-injection of Aurora A antibody at late G2 phase also inhibits separation of centriole pairs (Marumoto et al., 2003). These observations agree with studies in *Xenopus* showing that Aurora A (Eg2) can phosphorylate the kinesin-like protein XlEg5, a protein required for proper centrosome separation (Giet et al., 1999). Aurora A could regulate centrosome separation through the phosphorylation of the kinesin-like motor protein during prometaphase.

TPX2, the targeting protein for *Xenopus* kinesin-like protein 2, was identified as a binding partner, a substrate and also an activator of Aurora A (Eyers et al., 2003; Kufer et al., 2002). In mitosis, TPX2 is released by Ran-GTP from importin-α and importin-β (Gruss et al., 2001; Tsai et al., 2003). The liberated TPX2 then binds to Aurora A at the centrosome and targets it to the microtubules proximal to the pole (Kufer et al., 2002). Binding of TPX2 regulates the kinase activity of Aurora A both by stimulating Aurora A autophosphorylation at Thr295 and counteracting the activity of the protein phosphatase

PP1 (Eyers et al., 2003). This activation of Aurora A is important for spindle assembly *in vitro* (Roghi et al., 1998).

In HeLa cells, Aurora A level and kinase activity increase during late G2 to M phase (Bischoff et al., 1998). Aurora A activity is highly regulated during mitosis by both phosphorylation/dephosphorylation and degradation. Phosphorylation stimulates kinase activity of Aurora A. Three phosphorylation sites have been identified in *Xenopus* Aurora A by mass spectrometry – Thr295, Ser53 and Ser349 (Littlepage et al., 2002). Thr295 is in a conserved activation loop for all Aurora kinases and has also been identified as an autophosphorylation site in Ipl1 (Cheeseman et al., 2002). Ser53 is in the N-terminal A-box, which is essential for Aurora A degradation mediated by APC/C complex during mitotic exit. Phosphorylation of Ser53 makes the kinase resistant to degradation (Littlepage and Ruderman, 2002). Phosphorylation of Ser349 is not essential for catalytic activation, but S349D mutants can block kinase activation. Thus, it may have a regulatory role for the activation of Aurora A.

In HeLa cells, low levels of Aurora A have also been found at the spindle mid-zone during late mitosis (Marumoto et al., 2003; Sugimoto et al., 2002). The dynamic localization pattern of Aurora A suggests that it might be involved in various mitotic events, not just limited to mitotic spindle formation. Research has shown that Aurora A can phosphorylate CENP-A at Ser7 and is involved in metaphase chromosome alignment (Kunitoku et al., 2003). In addition, both the inhibition and overexpression of Aurora A can lead to multinucleated cells (Marumoto et al., 2003; Meraldi et al., 2002). These results suggest that successful cytokinesis is dependent upon the proper timing of Aurora A activation and inactivation.

1.1.2 Aurora B – the Equatorial Aurora

Aurora B kinases are chromosomal passenger proteins that are essential for multiple critical processes during mitosis, such as chromosome condensation and segregation, chromosome biorientation, and cytokinesis. Similar to the localization pattern of other chromosomal passenger proteins, Aurora B kinases associate with centromeres during prometaphase and metaphase and translocate to the central spindle at the metaphase/anaphase transition. This localization pattern is consistent in mammalian cells, *C. elegans* and *Drosophila* (Adams et al., 2001b; Schumacher et al., 1998b; Terada et al., 1998). MKLP2, a kinesin like protein, is essential for relocation of Aurora B from centromeres to the central spindle at the metaphase/anaphase transition (Gruneberg et al., 2004). In budding yeast, Ipl1 localizes to kinetochores and the mitotic spindle during mitosis (Buvelot et al., 2003).

During prometaphase and metaphase, an important mission for mitotic chromosomes is to establish bipolar attachment to microtubules emanating from opposite spindle poles. This bipolar attachment is essential for positioning the chromosome at the metaphase plate and proper chromosome segregation. Pioneering studies performed more than 30 years ago implicated mechanical tension as a key regulation factor of bipolar attachment (Nicklas and Staehly, 1967). At the molecular level, it was first found that Ipl1 could destabilize single-pole attachments of sister chromatids (Tanaka et al., 2002). It has been proposed that, when a chromosome is bipolarly attached, the tension created by the mitotic spindle can stretch the sister kinetochores apart. In this case the sister kinetochores are pulled away from the inner centromeres, where Aurora B is found. Thus, the attached microtubules are no longer susceptible to the destabilizing activity of Aurora

B and remain stably bound to the kinetochores. In more complex eukaryotes, Aurora B kinases have an important role in regulating kinetochore-microtubule interactions. Recent studies have shown that the N-terminus of Ndc80 contains key Aurora B phosphorylation sites that cause microtubule detachment when phosphorylated (DeLuca et al., 2006).

Aurora B also plays a central role in chromosome condensation. Aurora B kinases are responsible for phosphorylation of histone H3 on S10 and S28 (Adams et al., 2001c; Giet and Glover, 2001; Goto et al., 2002; Hsu et al., 2000). These histone modifications are thought to help mitotic chromatin condensation (Goto et al., 1999; Hendzel et al., 1997). Activation of Aurora B kinase at the centromeres also leads to displacement of HP1 from mitotic chromosomes, which might be required for chromosome segregation (Fischle et al., 2005; Hirota et al., 2005).

Overexpression of a catalytically inactive form of Aurora B prevents the final steps of cytokinesis in mammalian cells (Terada et al., 1998). A similar phenotype was also produced by exogenous expression of truncated INCENP that lacks the Aurora B binding/activating domain (Adams et al., 2000; Mackay et al., 1998). Current research has shown that Aurora B phosphorylates MgcRacGAP, a human GTPase-activating protein, in the midbody of mammalian cells (Minoshima et al., 2003). This phosphorylation significantly changes the specificity of MgcRacGAP from an activating protein for Rac1/Cdc42 GTPase to an activator of RhoA. RhoA regulates actin polymerization and is essential for the completion of cytokinesis (Drechsel et al., 1997; Kishi et al., 1993). Therefore, it is possible that Aurora B regulates cytokinesis through a RhoA pathway by the phosphorylation of MgcRacGAP.

As the enzymatic core of the chromosomal passenger complex, Aurora B can interact with other members of the complex – INCENP, Survivin and Borealin (Adams et al., 2000; Bolton et al., 2002; Gassmann et al., 2004; Honda et al., 2003). Recent studies suggest that two different kinds of chromosomal passenger complexes exist during mitosis: one containing all four chromosomal passenger complex (CPC) members, that functions during chromosome alignment and cytokinesis, the other consisting of INCENP and Aurora B, which is responsible for the modification of histone H3 (Gassmann et al., 2004).

Both INCENP and survivin have a role in activation of Aurora B kinases. INCENP is the most studied binding partner of Aurora B. It has a highly conserved carboxy-terminal domain – the IN-box, which is responsible for Aurora B binding (Honda et al., 2003). INCENP is phosphorylated by Aurora B in the IN-box and this phosphorylation can enhance the activity of the kinase (Bishop and Schumacher, 2002).

The expression and activity of Aurora B in mammalian cells are also cell cycle regulated. Expression peaks at the G_2-M transition and kinase activity is maximal during mitosis (Bischoff et al., 1998; Terada et al., 1998). Aurora B activity is also spatially regulated through the interaction with INCENP and survivin at different locations in different stages of mitosis. Aurora B is degraded by the APC/C complex in late mitosis and in G_1, which is dependent on its C-terminal D-box (Stewart and Fang, 2005).

1.2 *DICTYOSTELIUM*

1.2.1 Overview of *Dictyostelium*

The cellular slime mold, *Dictyostelium discoideum,* is a simple eukaryotic microorganism that lives in the soil. *Dictyostelium* amoebae grow and divide as individual cells as long as a food source is available. When challenged by adverse conditions such as starvation, thousands of amoebae interact to form multicellular structures. Up to 100,000 cells signal each other by releasing the chemoattractant cAMP and aggregate together by chemotaxis to form a finger structure. Then they thoroughly integrate into a multicellular structure – a migrating slug that can move slowly towards light and along a temperature gradient. Finally through a process called culmination, the cells differentiate into a fruiting body consisting of a cellular stalk supporting a mass of spores (Bonner, 1967; Loomis, 1982). Because of its relatively simple development process, *Dictyostelium* has been used as a classic model system for developmental biology.

Dictyostelium is also a powerful system for basic biomedical research in cell biology. Since the behavior of the amoebae resembles the behavior of ameboid mammalian cells, *Dictyostelium* has unique advantages for studying many fundamental cellular processes, such as cytokinesis, cell motility, phagocytosis and membrane trafficking (De Lozanne and Spudich, 1987; Larochelle et al., 1996; O'Halloran and Anderson, 1992).

Dictyostelium has a relatively simple genome of 34Mb, which has been fully sequenced. *Dictyostelium* is haploid and the genome contains many genes that

are homologous to those in more complex eukaryotes and are missing in *S. cerevisiae*. Disruption of a certain gene can be carried out at high frequency by homologous recombination (De Lozanne, 1987). It is also possible to create a library of random plasmid insertions by Restriction Enzyme-Mediated Integration (REMI) (Kuspa and Loomis, 1992). Since *Dictyostelium* cells can be grown either on nutrient agar plates feeding on bacteria, or in shaken suspension in axenic medium, it is fairly easy to culture cells in large scale to carry out biochemical research. All these features make *Dictyostelium* an excellent model system for functional analysis of many different subcellular processes.

1.2.2 Cell Cycle of *Dictyostelium*

Dictyostelium cells double every 3-4 hours when grown on bacteria. When grown in axenic medium, they double every 8-10 hours (Watts and Ashworth, 1970). *Dictyostelium* cells do not have a G_1 phase. After M phase, the cells proceed rapidly into a very short S phase of 30 minutes or less. In contrast, the G_2 phase is lengthy in *Dictyostelium*, lasting 6 hours or more (Weijer et al., 1984). The M phase usually takes about 10 minutes and is divided into two equal portions by the start of anaphase (Roos and Camenzind, 1981).

As in other protists, algae and fungi (Heath, 1974), the *Dictyostelium* nuclear envelope remains mostly intact during mitosis (Moens, 1976). However, the spindle dynamics of *Dictyostelium* cells are quite similar to that of more complex eukaryotes. During interphase, the spindle pole body separates from the nucleus to a distance of 0.7μm (Moens, 1976). When cells enter prophase, most cytoplasmic microtubules are

disassembled, the spindle pole body returns to the nuclear envelope and docks onto the nuclear membrane. Later in prophase/prometaphase, the spindle pole body duplicates and begins to separate, thereby initiating the formation of the mitotic spindle. As it elongates, the newly formed spindle changes from a dot at the nuclear envelope to a short rod of microtubules across the nucleus in prometaphase/metaphase (Roos et al., 1984). When the length of the spindle reaches about 2μm, all chromosomes are aligned at the equatorial plate, forming a cluster around the bundle of the central spindle with their centromeric ends pointing inward. Sister chromatids segregate synchronously at the metaphase/anaphase transition. In anaphase, the central spindle elongates further and the chromosomal microtubules shorten, which brings kinetochores proximal to spindle poles. During anaphase and telophase, *Dictyostelium* cells have a very prominent central spindle. The central spindle is disassembled before the end of cytokinesis and there is no midbody structure found in *Dictyostelium* (McIntosh et al., 1985; Roos et al., 1984).

A unique feature of *Dictyostelium* cell division is that the duplication and separation process of the centrosome is different from that of vertebrate cells. In interphase, the *Dictyostelium* centrosome is a box-shaped structure comprised of three major layers. Structural duplication takes place during prophase, as opposed to G_1/S phase in vertebrate cells. In prometaphase, it separates into two mitotic centrosomes via a lengthwise splitting process. The two outer layers peel away from each other and spindle microtubules are nucleated from their inner surfaces. At the metaphase/anaphase transition, the centrosomes docked in the nuclear envelope start to curl outwards and are displaced from the nuclear envelope. Finally they fold back into an interphase structure

during telophase (Ueda et al., 1999). Thus, in each cell cycle the centrosome undergoes an inside-out/outside-in reversal of its layered structure.

Although the morphology of the *Dictyostelium* centrosome and mitotic spindle has been studied in detail, information regarding underlying cell cycle regulation mechanisms is lacking. In *Dictyostelium*, the DNA sequence of centromeres has yet to be defined; the chromosomal passenger complex has not been fully characterized; and mitotic kinases, such as Aurora and Polo kinases, have not been studied. However, many cytokinesis regulation factors have been identified and studied in *Dictyostelium*. Examination of essential mitosis regulators in *Dictyostelium*, such as Aurora kinases, will improve our understanding of its cell division and establish more connections between mitosis regulation and cytokinesis regulation.

1.2.3 Regulation Factors for Cytokinesis in *Dictyostelium*

Since *Dictyostelium* has a very distinct stage of cytokinesis closely resembling cytokinesis of vertebrate cells, it has been used as a model system to study the last step of M phase.

Cytokinesis starts with the initiation of the cleavage furrow at the equatorial plate and ends with abscission of the cytoplasmic bridge connecting the two daughter cells. The driving force of the cleavage furrow is the actomyosin contractile ring. Using *Dictyostelium*, researchers found that disruption of myosin heavy chain yields cells that are unable to complete cytokinesis (De Lozanne and Spudich, 1987). Successful cytokinesis in *Dictyostelium* also requires coordination of actin bundling mediated by cortexillin and Rac1 (Faix et al., 2001), clathrin-dependent membrane trafficking

processes (O'Halloran, 2000), dynamin (Wienke et al., 1999), and cortex tension regulation conducted by RacE (Gerald et al., 1998). In addition, many other proteins have been shown to be important for cytokinesis including LvsA (Kwak et al., 1999), DGAP1 (Faix and Dittrich, 1996), coronin (de Hostos et al., 1993), dynacortin (Robinson and Spudich, 2000) and pats1 (Abysalh et al., 2003).

To coordinate cytokinesis with the rest of mitosis, it must be highly regulated by upstream factors from mitosis. However, the mechanism of cytokinesis initiation remains unclear. Several key questions, such as how cytokinesis is induced, how the cleavage furrow is positioned and initiated, have not been fully addressed. Since Aurora kinases are essential for multiple stages of mitosis, including cytokinesis, and Aurora B kinases are localized to the central spindle from anaphase to the end of cytokinesis, these proteins are possible candidates that act as upstream regulators of cytokinesis.

1.3 SIGNIFICANCE OF STUDYING *DICTYOSTELIUM* AURORA KINASE

As one of the most important regulatory proteins for mitosis and cytokinesis, the exact role of Aurora kinases in cytokinesis initiation is still elusive. In *Dictyostelium*, Aurora kinases have yet to be examined. Recent studies of another chromosomal passenger protein – DdINCENP – suggests that the chromosomal passenger complex is essential for cytokinesis in *Dictyostelium* (Chen et al., 2006). As the potential binding partner of DdINCENP, *Dictyostelium* Aurora kinase could also be essential for cytokinesis or even for other stages of mitosis. To elucidate its roles in mitosis and cytokinesis, I have investigated the role of Aurora kinase in *Dictyostelium*.

In this dissertation, I show that there is only one Aurora kinase in *Dictyostelium*, which we named DdAurora. In contrast to the single Aurora kinase in yeast, which only shows characteristics of Aurora B, DdAurora shows sequence characteristics of both Aurora A and Aurora B. The *Dictyostelium* homolog is the first Aurora kinase that has been identified with sequence characteristics of both Aurora A and Aurora B. DdAurora may represent the function of ancestral Aurora kinases in less complex eukaryotes in which Aurora A and B have not diverged. My investigation of the localization of the endogenous DdAurora at spindle poles, centromeres and the central spindle suggests that DdAurora functions similar to both Aurora A and Aurora B and it is essential for mitosis and cytokinesis. Co-localization and co-immunoprecipitation assays demonstrate that DdAurora binds to DdINCENP and forms a chromosomal passenger complex. I confirmed that the C-terminal IN-box domain of DdINCENP is essential for DdAurora binding and localization of DdAurora to the central spindle. I also show that the kinesin-6 related protein Kif12 is responsible for the localization of the DdAurora/DdINCENP complex to the central spindle. Finally, I show that Myosin II is required for proper localization of the DdAurora/DdINCENP complex to the cleavage furrow in cytokinesis. To further explore the function of this kinase in mitosis, I identified two potential binding partners or substrates of DdAurora – topoisomerase B and HspA by large-scale immunoprecipitation. The current study provides new insights into Aurora kinase function during mitosis and cytokinesis in eukaryotic cells.

Chapter 2 DdAurora Shares Properties of both Auroras A & B

2.1 INTRODUCTION

Since the discovery of Aurora kinases in different organisms, the proteins have been shown to function during multiple stages of cell division. From chromosome condensation and separation in prophase (Berdnik and Knoblich, 2002; Glover et al., 1995; Hannak et al., 2001; Schumacher et al., 1998a) to the very end of cytokinesis (Marumoto et al., 2003; Meraldi et al., 2002; Terada et al., 1998), Aurora kinase activity covers almost every crucial transition stage of M phase.

Most metazoans have both Aurora A and Aurora B kinases to mediate mitosis. Although Aurora A and Aurora B kinases have very distinct localization and function during cell division, they have many things in common. First, each has a highly conserved kinase domain that covers more than 75% of the entire protein sequence. The two kinases differ only at the short N-terminus tail. Second, the expression level and activity of both kinases are cell cycle regulated, peaking at the G_2-M transition (Bischoff et al., 1998). Both kinases are degraded by the APC/C complex at the end of mitosis through the mediation of the D-box sequence at their C-termini (Littlepage and Ruderman, 2002; Stewart and Fang, 2005). Third, both Aurora A and B are thought to function during cytokinesis and their localization overlaps at the central spindle and midbody. These common characteristics suggest that Aurora A and Aurora B could be derived from the same ancestral kinase.

Yeast contains one Aurora kinase, Ipl1 in budding yeast and Ark1 in fission yeast (Chan and Botstein, 1993; Petersen et al., 2001). Both have been shown to be mainly involved in chromosome segregation and cytokinesis (Francisco and Chan, 1994; Leverson et al., 2002; Petersen and Hagan, 2003), processes that are usually regulated by Aurora B kinase. Ark1 mutant cells exhibited a minor defect in spindle formation (Petersen et al., 2001). However, investigators were unable to detect Ark1 on the spindle pole body from prometaphase to telophase. Because the yeast mitotic spindle formation process is quite different from that of mammalian cells, Aurora A may not be essential for spindle formation in yeast cells.

Aurora kinase has not been well characterized in other simple eukaryotes. *Dictyostelium* is an excellent model system to study mitosis and cytokinesis in eukaryotic cells. Study of Aurora kinase in *Dictyostelium* should improve our understanding of the mitotic kinase function. Here I characterize DdAurora, the single Aurora kinase in *Dictyostelium*. I examined the localization of this protein during mitosis and found it to be the first single Aurora kinase that exhibits properties of both Aurora A and B kinases. In addition, I found that overexpression of a kinase inactive form of DdAurora causes dominant negative effects during cytokinesis. These findings suggest that DdAurora represents an ancestral kinase for both Aurora A and Aurora B and thus, may have essential roles for several critical steps of M phase.

2.2 RESULTS

2.2.1 Identification of *Dictyostelium* Aurora

Searching the *Dictyostelium* genomic database with protein sequences of Aurora kinases from different species, I identified the only potential Aurora gene in *Dictyostelium*, which I named DdAurora (entry DDB0216254 in http://dictybase.org; Genebank accession XP_641803). The sequence of the *DdAurora* gene encodes a protein with a molecular weight of 43kDa with a conserved Serine/Threonine protein kinase domain. DdAurora shows high sequence similarity to Aurora kinases from other organisms, including both Aurora A and Aurora B kinases (Figure 2.1). DdAurora is 48% identical in amino acid sequence to Human Aurora B and 44% identical to Human Aurora A. This is the first identification of an Aurora kinase with specific sequence similarity to both Aurora A and Aurora B kinases. DdAurora has a signature motif (DFGWS*XXXXXXX*R*X*T*X*CGT*X*DYLPPE) in the activation loop specific for Aurora kinases in the catalytic domain (Cheetham et al., 2002) and a D-box (LL*XXX*P*XX*R*XX*L*XXXXX*HPW) near the C-terminus that is common to all Aurora kinases (Castro et al., 2002; Stewart and Fang, 2005). DdAurora also has a potential truncated A-box (P**S**XXXQRVXXQ) near the N terminus (Figure 2.1), which is a specific structural feature of mammalian and *Xenopus* Aurora A and is required for Cdh1-dependent destruction of Aurora A. Phosphorylation of the consensus Serine site in this motif is able to block destruction of Aurora A (Littlepage and Ruderman, 2002).

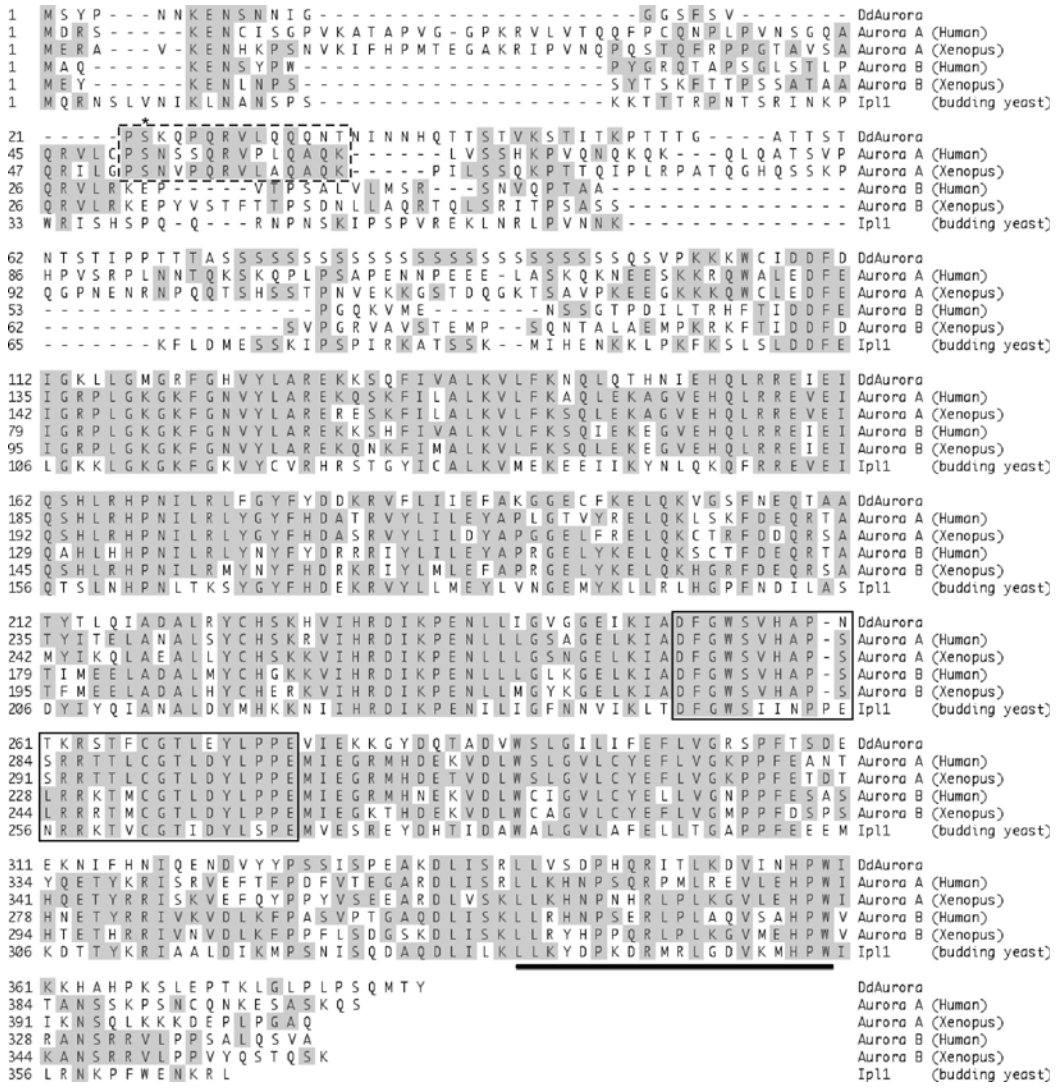

Figure 2.1 Alignment of DdAurora with Aurora kinases from other organisms.

DdAurora shows high sequence similarity to both Aurora A and Aurora B kinases. DdAurora has a potential N-terminal A-box (P**S***XXX*QRV*XX*Q, framed in dashed box), which is essential for Aurora A destruction. The conserved phosphorylation site – Serine22 is shown with an asterisk. This consensus site is known to be phosphorylated in vertebrates to block Cdh1-dependent destruction of Aurora A. The signature motif (DFGWS*XXXXXXX*R*X*T*X*CGT*X*DYLPPE) in the activation loop is framed and the D-box (LL*XXX*P*XX*R*XX*L*XXXXX*HPW) near the C-terminus is underlined. All Aurora kinases have these two motifs to regulate their activation and degradation respectively.

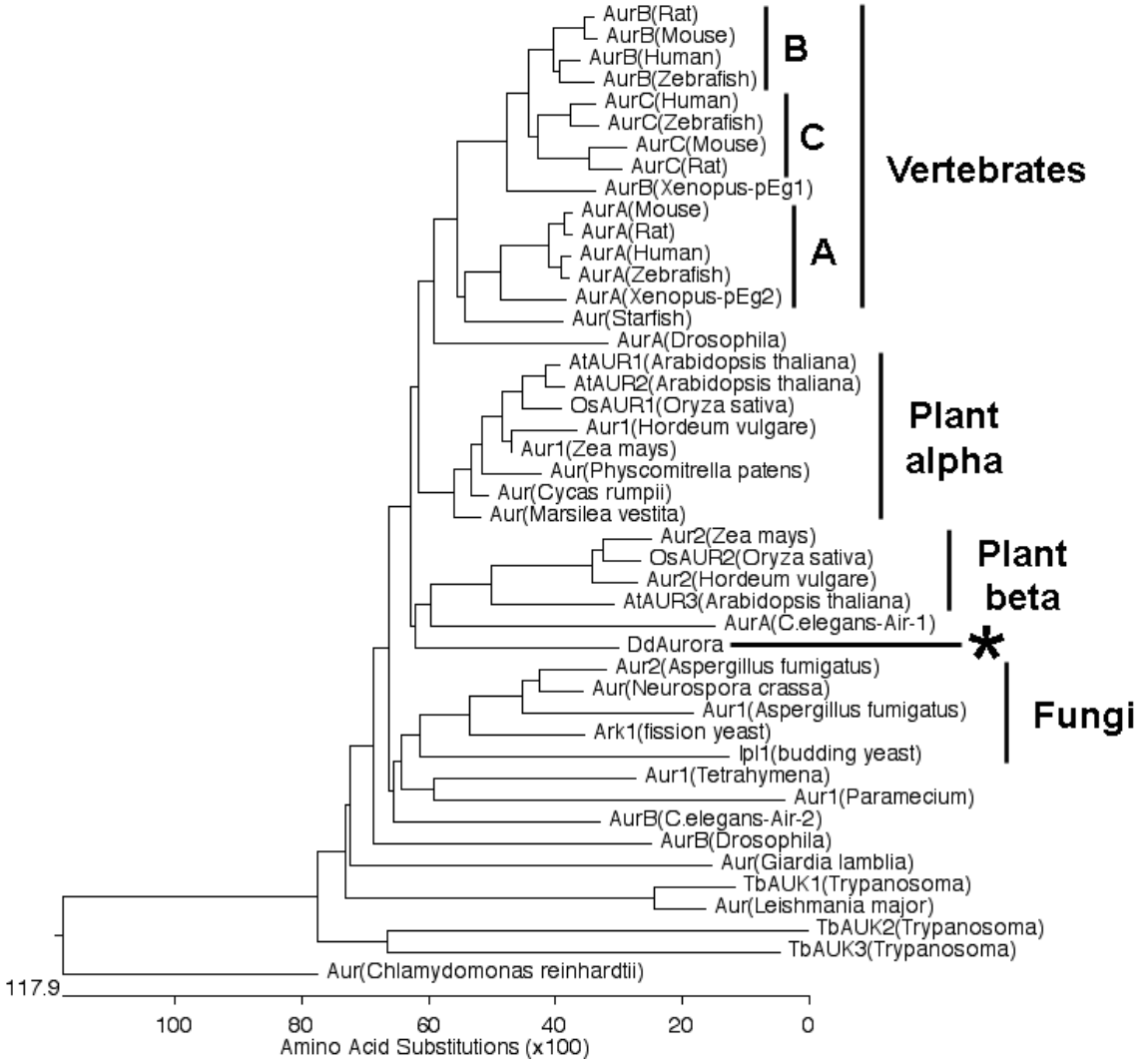

Figure 2.2 Phylogenetic tree of Aurora kinases from different species.

Aurora kinase sequences were aligned by Clustal W method in MegAlign and a complete phylogenic tree was generated. Aurora kinases in vertebrates are divided into three clades of Aurora A, B and C sub-families. DdAurora (asterisk) forms a clade with Aurora kinases from the plant kingdom, which branches before the divergence of Aurora A, B and C sub-families. It strongly suggests that DdAurora represents a progenitor of both Aurora A and Aurora B in more complex eukaryotes.

In order to construct a comprehensive phylogenetic tree, different genome databases were searched for Aurora kinases from several organisms, including mammals, cold-blooded vertebrates, plants, fungi and protists. The kinase domain sequences of these proteins were aligned by Clustal W method in MegAlign and a complete phylogenic tree was generated (Figure 2.2). From the phylogenetic tree we can see that Aurora kinases in vertebrates are divided into three clades of Aurora A, B and C sub-families. The *Dictyostelium* Aurora kinase branches off near the base of the animal and plant groups. Interestingly, DdAurora is more similar to the animal and plant kinases than the Aurora proteins from fungi. Since it is generally thought that *Dictyostelium* diverged from the lineage leading to animal cells before the fungi but after plants, the analysis is suggestive that DdAurora may resemble most closely the ancestral Aurora kinase in the common ancestor of plants, fungi and animals. If this is the case, this ancestral Aurora kinase might have characteristics of both Aurora A and Aurora B kinases.

2.2.2 Localization of GFP-DdAurora in M Phase

Since Aurora A and Aurora B have very distinct localizations during mitosis in animal cells, I investigated where the single Aurora kinase would localize in *Dictyostelium* cells. To examine the cellular localization of DdAurora, a GFP-DdAurora construct was generated and transformed into wild type *Dictyostelium* cells. The localization of GFP-DdAurora during mitosis was examined by fluorescence microscopy of living cells.

In interphase, most of the GFP-DdAurora was diffuse throughout the entire cytoplasm (Figure 2.3a). When cells entered mitosis, GFP-DdAurora was first found at spindle poles during prometaphase (Figure 2.3b). GFP-DdAurora formed bright foci at both spindle poles, making it easier to identify mitotic cells out of thousands of interphase cells. Additionally, GFP-DdAurora was present at the metaphase plate during metaphase (Figure 2.3c). At the metaphase/anaphase transition, GFP-DdAurora localized to the central spindle and remained there during telophase and early cytokinesis (Figure 2.3d-f). Interestingly, GFP-DdAurora appeared at the cortex of the cleavage furrow only at a very late stage of cytokinesis (Figure 2.3g). This is in marked contrast to DdINCENP, which is known to localize to the equatorial cortex region early in telophase when the cleavage furrow just begins to form (Chen et al., 2006). After cytokinesis finished, GFP-Aurora persisted at the breaking point of the cytoplasm bridge for a time (Figure 2.3i).

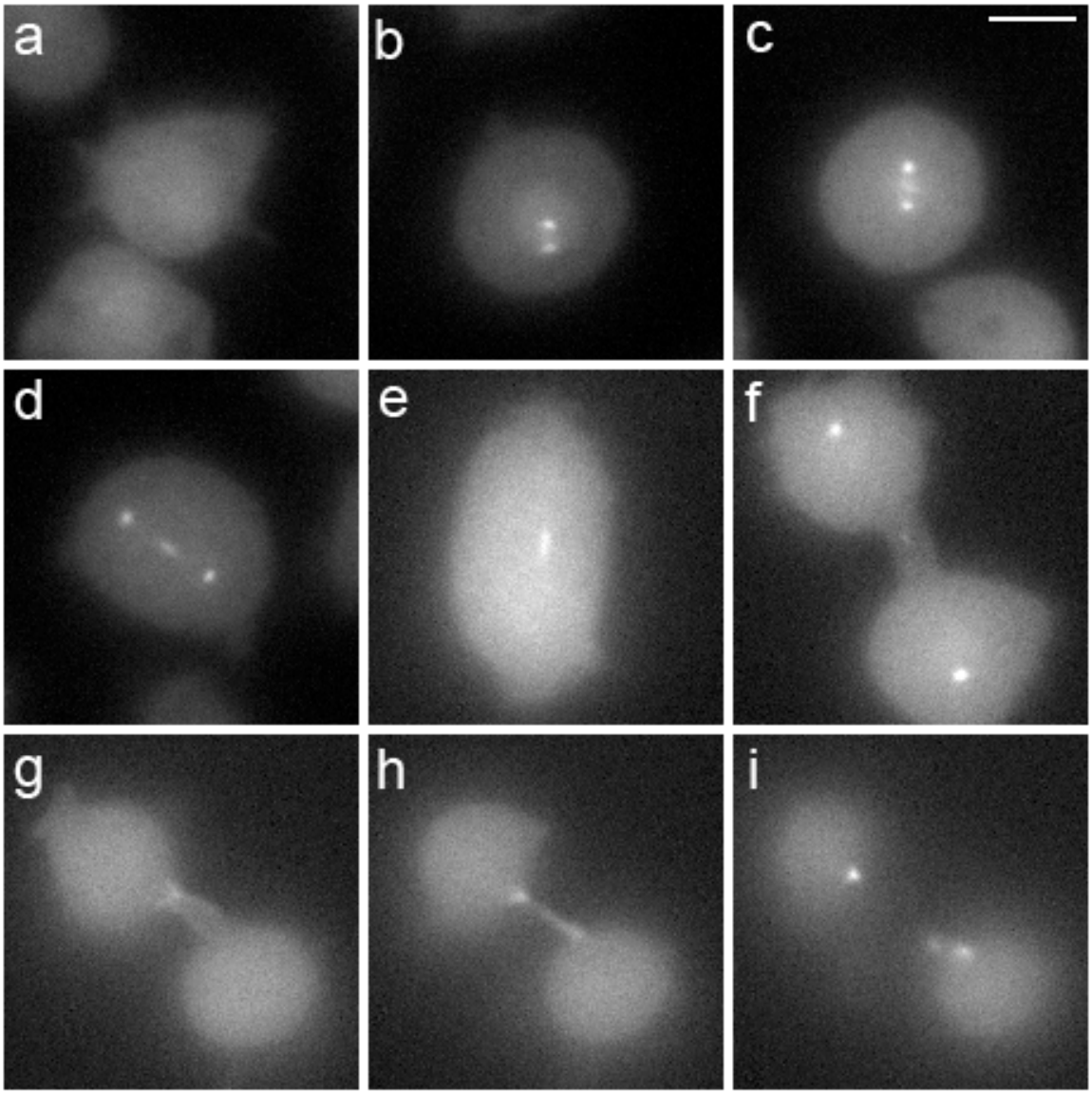

Figure 2.3 GFP-DdAurora localization in different stages of M-phase.

Fluorescence images of a live wild-type cell expressing GFP-DdAurora. (a) In interphase, GFP-DdAurora was diffuse in the cytoplasm. (b) During prometaphase, GFP-DdAurora concentrated at spindle poles. (c) During metaphase, GFP-DdAurora also localized to the metaphase plate in addition to spindle poles. (d-f) From anaphase to early cytokinesis, GFP-DdAurora localized to the spindle poles and the central spindle. (g) GFP-DdAurora localized to the cleavage furrow at a very late stage of cytokinesis. (h) Cytoplasmic bridge localization of GFP-DdAurora in late cytokinesis. (i) GFP-DdAurora persisted at the breaking point of the cytoplasm bridge for a time after cytokinesis. Bar, 5μm.

The localization pattern of DdAurora suggests that, unlike Aurora kinases from other species, the single *Dictyostelium* Aurora kinase exhibits both Aurora A and Aurora B characteristics during mitosis.

2.2.3 Endogenous DdAurora Localizes at the Spindle Poles and Central Spindle

To determine whether the distribution of GFP-DdAurora mirrored that of the endogenous protein, I raised polyclonal antibodies against DdAurora. Affinity purification of these antibodies enabled me to examine the localization of endogenous DdAurora during mitosis.

Using immunofluorescence microscopy, I determined the localization of DdAurora in relation to that of DdCP224, a *Dictyostelium* centrosome resident protein (Graf et al., 2000). A monoclonal antibody against DdCP224 was used to observe the localization of the endogenous DdCP224 simultaneously with DdAurora. After close examination of their respective distributions (Figure 2.4), I found that from prometaphase to early anaphase, the endogenous DdAurora localized to spindle poles. However, rather than displaying overlapping co-localization with DdCP224, DdAurora localized in close proximity but distal to DdCP224 (Figure 2.4a-c). Since DdCP224 is known to colocalize with γ-tubulin exactly at the spindle poles (the microtubule nucleation center) (Graf et al., 2000), I concluded that DdAurora may localize to the outer sides of spindle poles from metaphase to early anaphase. Interestingly, this outer polar localization of DdAurora disappeared in late anaphase to be replaced by a dot of DdAurora on the inner side of the spindle poles (Figure 2.4d-e). This localization is discussed in more detail in the next section.

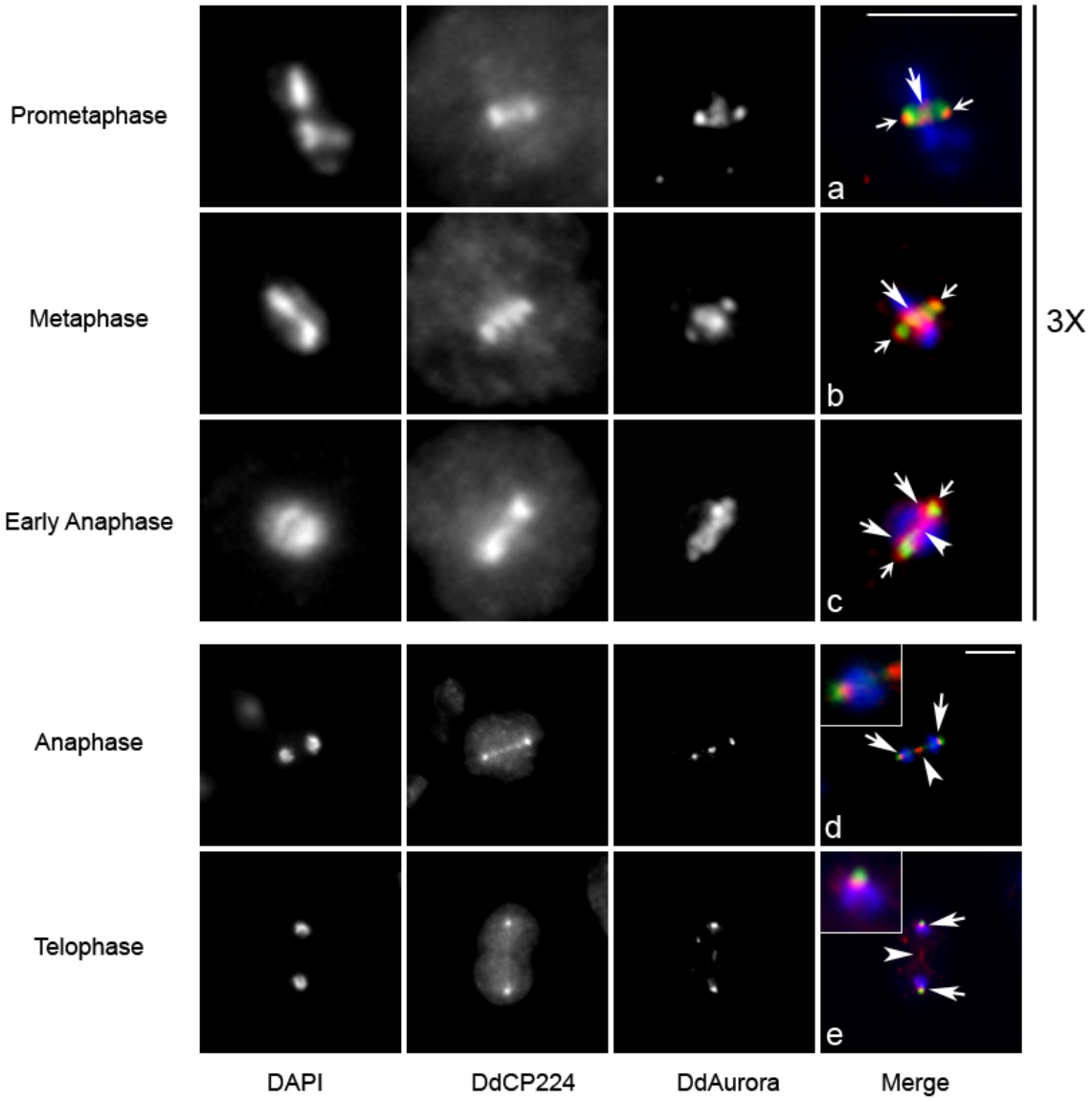

Figure 2.4 Co-localization of endogenous DdAurora with DdCP224 in different stages of mitosis.

In the merged images, DNA is shown in blue, DdAurora in red and DdCP224 in green. (a-b) In prometaphase and metaphase, DdAurora localized to both spindle poles (shown by small arrows) and the metaphase plate (shown by arrows). The metaphase plate localization will be shown to be the centromeric localization in the following section (Figure 2.5). The centromeric localization of endogenous DdAurora in metaphase is much stronger than that of GFP-DdAurora shown in Figure 2.3c. Inserts show enlarged image of the mitotic spindle, DdAurora localized in close proximity but distal to DdCP224. It probably localized on the outer side of the spindle poles. (c) In early anaphase, DdAurora localized simultaneously to spindle poles (shown by small arrows), centromeres (shown by arrows) and the central spindle (shown by arrowheads). (d-e) In late anaphase and telophase, a portion of DdAurora kept associated with centromeres (shown by arrows) and showed as inner polar dots following the migration of spindle poles shown by DdCP224 immunostaining (green). The outer polar localization of DdAurora was not observed in these stages. DdAurora still localized to the central spindle (shown by arrowheads). Bar, 5μm. The top panel is enlarged 3x magnification.

As mentioned previously in chapter 1, the *Dictyostelium* centrosome has a unique structure and distinct duplication cycle (Ueda et al., 1999). At the metaphase/anaphase transition, centrosomes docked at the nuclear envelope start to curl outwards and fold back into a three-layered structure. If DdAurora is bound on the outer surface of the centrosome, it may become enfolded by the curled centrosome and be no longer accessible to antibodies at this stage. Alternatively, it is possible that DdAurora leaves the centrosome or is degraded at this location once *Dictyostelium* cells enter anaphase.

In addition to the polar localization, the endogenous DdAurora could also be detected at the central spindle from prometaphase to cytokinesis. However, the central spindle localization of endogenous DdAurora in metaphase is much stronger than that observed with GFP-DdAurora. This may indicate that endogenous DdAurora associates with centromeres better than GFP-DdAurora.

2.2.4 Endogenous DdAurora Localizes to Centromeres even after the Metaphase to Anaphase Transition

Careful comparison of cells at different stages revealed that, while DdAurora was found on the outer side of the metaphase poles, it was later found on the inner side of the spindle poles in anaphase and telophase. Figure 2.4 (a-b) shows that endogenous DdAurora (red) was on the outside of the prometaphase and metaphase poles labeled by the centrosomal marker DdCP224 (green). In contrast, figure 2.4 (d-e) shows that endogenous DdAurora was on the inner side of the centrosomes in anaphase and telophase. This inner side localization of DdAurora is reminiscent of that of HcpA, a centromere-localized protein homologous to mammalian HP1 (Kaller et al., 2006). This

suggested the possibility that DdAurora remains bound to centromeres after metaphase. This possibility would go against the conventional view that Aurora B abandons the centromeres with the onset of anaphase. Therefore, to explore this issue in detail I decided to compare the localization of endogenous DdAurora and HcpA-GFP in wild-type cells (Figure 2.5).

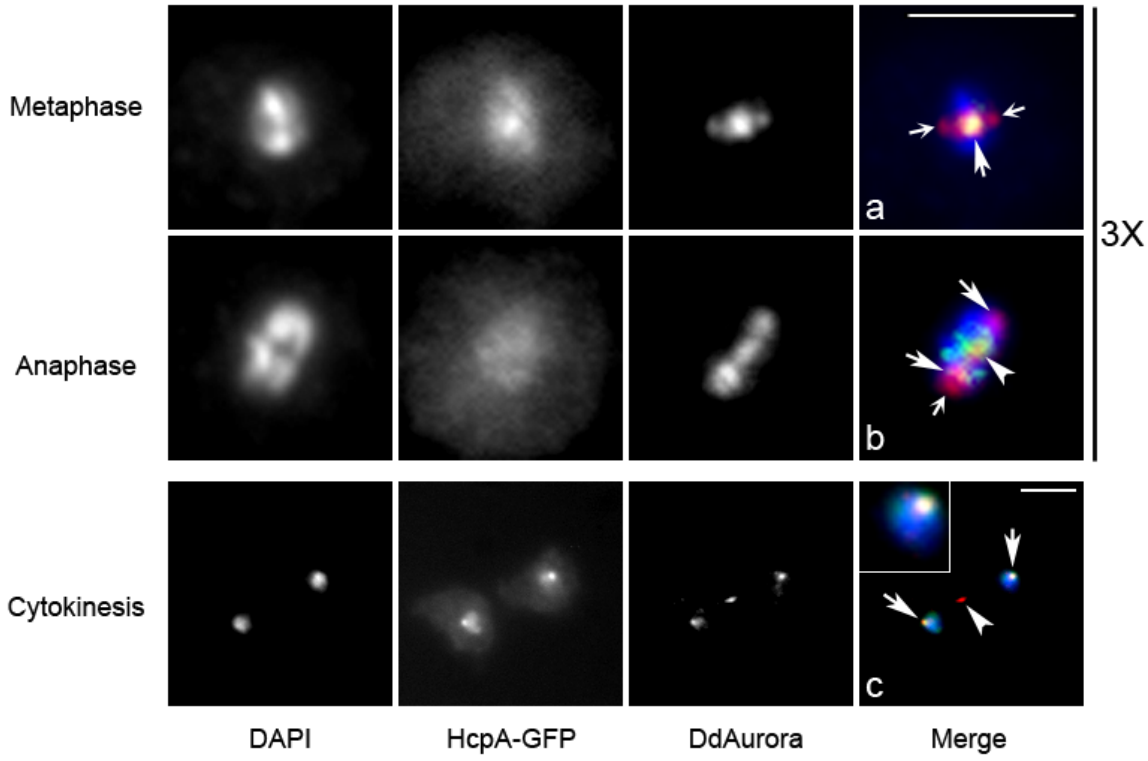

Figure 2.5 Co-localization of DdAurora with HcpA-GFP in different stages of mitosis.

In the merged images, DNA is shown in blue, DdAurora in red and HcpA-GFP in green. (a) In metaphase, DdAurora localized to spindle poles (shown by small arrows) and the metaphase plate. The metaphase plate DdAurora colocalized with HcpA-GFP extensively (shown by arrows), suggesting that DdAurora localizes to the centromeres in metaphase. (b) In anaphase, DdAurora was found at the central spindle (shown by arrowhead) in addition to the spindle poles (shown by small arrows) and centromeres (shown by arrows). HcpA-GFP was diffusely distributed in the nucleus in anaphase, which suggests that HcpA might be displaced from the centromeres by activated DdAurora at the metaphase/anaphase transition. (c) In late telophase/cytokinesis, HcpA-GFP could be observed again at the centromeres and colocalized with centromeric DdAurora extensively (shown by arrows). DdAurora also localized to the central spindle (shown by arrowhead) in this stage. Bar, 5μm. The top panel is enlarged 3x magnification.

Examination of cells expressing HcpA-GFP and immunostained with anti-DdAurora antibodies revealed that DdAurora colocalized with HcpA-GFP at centromeres in metaphase (Figure 2.5a). The cell in metaphase contained all of its centromeres congregated as a single dot at the center of the metaphase spindle. In this cell, DdAurora colocalized with HcpA-GFP on the centromeres and was also present at the poles. As shown before, this polar localization is on the outer side of the spindle poles (Figure 2.4b).

Interestingly, the localization of HcpA-GFP was diffuse inside the nucleus of cells in anaphase (Figure 2.5b). This suggested that HcpA-GFP left the centromeres at this time. The mammalian homologue of HcpA, the protein HP1, is known to be displaced from mitotic chromosomes by Aurora B kinase during mitosis (Fischle et al., 2005; Hirota et al., 2005). Thus, it seems likely that HcpA is also displaced from centromeres by DdAurora at the beginning of anaphase.

Later in telophase HcpA-GFP was again visible at the centromeres (Figure 2.5c), which are closely apposed to the inner side of the spindle poles (Kaller et al., 2006). Remarkably, DdAurora clearly colocalized with HcpA-GFP at the centromeres of the telophase cell. These observations strongly indicated that DdAurora remained bound to the centromeres of the separating chromatids during anaphase and telophase. This phenomenon has not been observed in other species.

2.2.5 DdAurora is Important for Mitosis and Cytokinesis

To determine the role of DdAurora in mitosis and cytokinesis, I attempted to disrupt the DdAurora gene by homologous recombination. Transformation of a knockout

construct was performed multiple times in different cell lines. More than 100 transformants were examined by PCR to determine the disruption of the DdAurora gene and all transformants I tested were found to be non-homologous recombinants. Failure to generate a DdAurora null cell line indicates that DdAurora might be essential for *Dictyostelium*. It may be required for several critical steps of M phase, such as centrosome maturation and separation, chromosome segregation and cytokinesis.

In mammalian cells, overexpression of a catalytically inactive form of Aurora B prevents the last steps of cytokinesis (Terada et al., 1998). A similar kinase inactive form of DdAurora – DdAurora (K139R) was generated by site-directed mutagenesis and overexpressed in wild type cells. GFP-DdAurora (K139R) showed the same localization as GFP-DdAurora in metaphase, anaphase and telophase (Figure 2.6a-c). However, when mitotic cells progressed to cytokinesis, the cleavage furrow failed to initiate and contract while the elongation of the spindle was not affected. Because of the lack of coordination between these two events, the spindle disintegrated, which resulted in the failure of cytokinesis (Figure 2.6d-f). This observation suggests that the kinase activity of DdAurora is required for the initiation and progression of cytokinesis and spindle strength but not for mitotic spindle elongation.

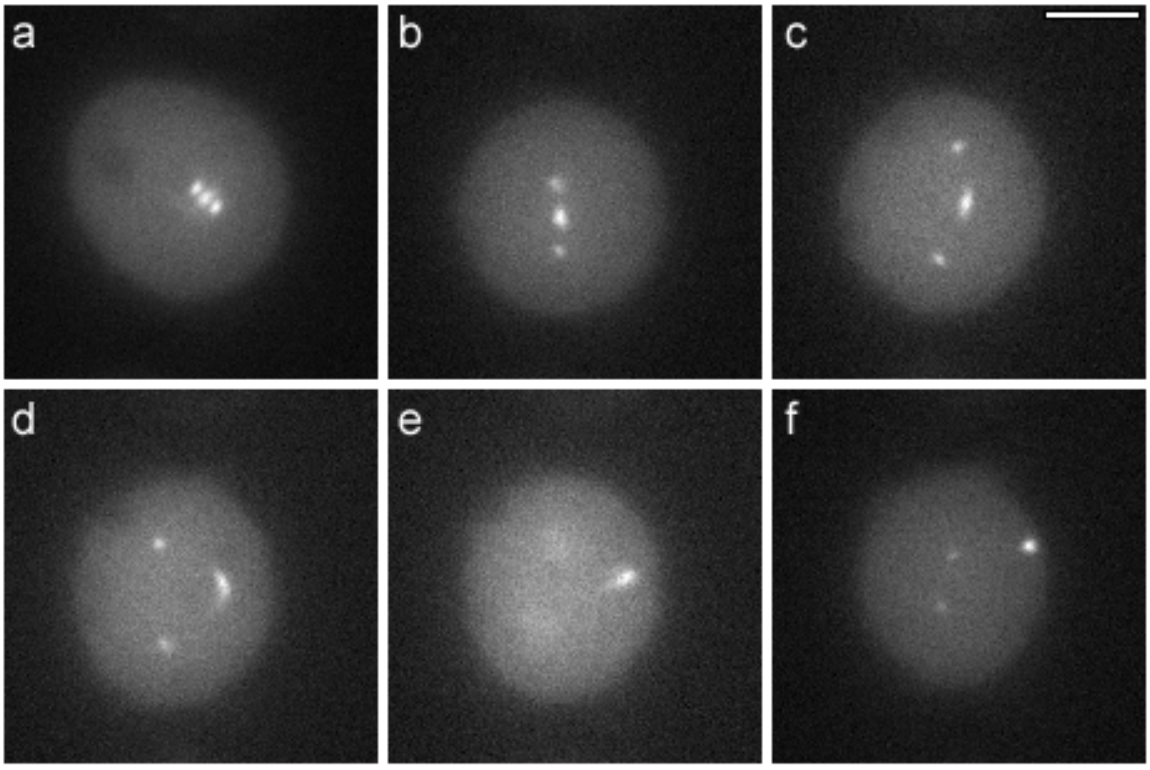

Figure 2.6 Overexpression of GFP-DdAurora (K139R) in wild-type cells.

Fluorescence images of a wild-type cell expressing kinase-inactive GFP-DdAurora (K139R). (a) In metaphase, GFP-DdAurora (K139R) localized to spindle poles and the metaphase plate. (b-c) From anaphase to telophase, GFP-DdAurora (K139R) localized to spindle poles and the central spindle. The GFP-DdAurora (K139R) displayed similar localization to that of GFP-DdAurora until this stage of mitosis. (d-f) When the cell progressed to cytokinesis, the cleavage furrow failed to initiate while the elongation of the spindle continued, which caused the spindle to bend and break in half. Bar, 5μm.

2.3 DISCUSSION

2.3.1 DdAurora, the First Single Aurora Kinase that Shows Characteristics of both Aurora A and Aurora B

Among all Aurora kinases that have been studied in various organisms, DdAurora is the first Aurora kinase that has been shown to have characteristics of both Aurora A and Aurora B simultaneously. Its similarity to both Aurora kinase sub-families is shown not only by sequence homology, but also by subcellular localization in different stages of mitosis in this chapter. The comprehensive phylogenetic tree of Aurora kinases from different species strongly suggests that DdAurora may represent an ancestral Aurora kinase that diverged into distinct Aurora A and Aurora B kinases in more complex metazoans. DdAurora may represent characteristics of Aurora kinases in simple eukaryotes, such as protists and algae. Mitosis is a complex process that requires cooperation of many different regulatory factors to function properly. In more complex eukaryotes, this process is mediated by both Aurora A and Aurora B in different stages. It will be interesting to learn how all these functions are conducted by a single Aurora kinase in simple eukaryotes. A possible explanation could be that DdAurora, the single Aurora kinase in *Dictyostelium*, is activated at different locations by specific binding partners/activators. So it is important to dissect the function of DdAurora at different subcellular localizations in different stages of mitosis.

2.3.2 DdAurora at the Spindle Poles

DdAurora localizes to spindle poles in prometaphase and metaphase, which represents characteristics of Aurora A kinase. In *Drosophila*, Aurora A mutants have a high frequency of monopolar mitotic spindles, which suggests a potential role for the kinase in centrosome separation (Glover et al., 1995). Lack of a DdAurora null cell line in *Dictyostelium* makes it difficult to define the function of DdAurora in centrosome separation and maturation. However, the inability to obtain a DdAurora null mutant indicates that it may be required for the initial steps of mitosis, such as centrosome separation and spindle assembly.

Overexpression of the kinase inactive form of DdAurora in wild-type cells did not show any defect in centrosome separation. The cells underwent prophase and metaphase normally compared to GFP-DdAurora expressing cells. They only showed defects in cytokinesis initiation and furrow contracting. On the other hand, overexpression of the kinase inactive form of DdAurora may affect spindle assembly by decreasing the mass of microtubules at the spindle, similar to what was shown in *C. elegans* (Hannak et al., 2001). This may explain why the spindle is so fragile in late telophase and cytokinesis when the inactive form of DdAurora is overexpressed. The spindle in metaphase and anaphase is so short that this defect may not be noticeable in early mitosis.

Co-localization of DdAurora with the centrosomal marker DdCP224 revealed that DdAurora is localized on the outer side of the spindle poles (Figure 2.4). In *Dictyostelium* the centrosome is embedded in the nuclear envelope during mitosis (Moens, 1976). Thus, one centrosomal surface remains in the cytosol while the other resides inside the nucleus. My observations suggest that there may be functional differences between these two

surfaces and that DdAurora may play a role on the cytosolic side of the centrosome during prometaphase and metaphase. In mammalian cells, Aurora A kinase was found to localize to centrosomes and the adjacent spindle microtubules (Sugimoto et al., 2002). It is possible that this pericentrosomal localization of Aurora A is equivalent to the localization of DdAurora on the outer portion of the centrosomes.

In vertebrates, TPX2 plays an important role in regulating Aurora A (Eyers et al., 2003; Kufer et al., 2002). An ortholog of TPX2 – TPXL-1 was found in *C. elegans* (Ozlu et al., 2005) but not in *Drosophila*. To determine whether a similar regulatory mechanism operates in *Dictyostelium* I searched the *Dictyostelium* genome database for sequences related to TPX2. However, I did not identify any *Dictyostelium* protein with any similarity to TPX2. Thus, the mechanism used to localize and regulate DdAurora at the spindle poles remains to be elucidated.

2.3.3 DdAurora at the Centromeres

A similarity between DdAurora and Aurora B kinases is their localization on centromeres during prometaphase and metaphase. This localization of Aurora B has been shown to be important for the regulation of proper microtubule attachment to kinetochores (DeLuca et al., 2006; Tanaka et al., 2002). Importantly, Aurora B leaves the centromeres at the onset of anaphase and redistributes to the central spindle together with INCENP and other chromosomal passenger proteins (Adams et al., 2001b; Schumacher et al., 1998b; Terada et al., 1998). In contrast, I found that DdAurora remains associated with centromeres even after the metaphase/anaphase transition and remains associated with centromeres for the rest of mitosis (Figure 2.5). Intriguingly, DdINCENP does leave

the centromeres like its animal counterpart during anaphase (Chen et al., 2006). Therefore, the association of DdAurora with the centromere appears to be independent of DdINCENP. This possibility is substantiated by the study of DdAurora localization in DdINCENP null cells (see Chapter 2). The continuous localization of DdAurora at centromeres may be important to maintain proper microtubule attachment during all stages of mitosis in *Dictyostelium*.

To demonstrate that DdAurora is found on centromeres I showed that DdAurora colocalized with the centromeric protein HcpA during early and late mitosis. In anaphase, HcpA is found diffuse in the nucleus, while DdAurora localizes to centromeres and the central spindle. This result rules out the possibility that HcpA is the binding partner of DdAurora at the centromeres. As the homolog of HP1, which is displaced from mitotic chromosomes by Aurora B in mammalian cells (Fischle et al., 2005; Hirota et al., 2005), HcpA may also be displaced from centromeres by DdAurora at the beginning of anaphase.

2.3.4 DdAurora at the Central Spindle

As a member of the chromosomal passenger complex, Aurora B translocates from centromeres to the central spindle at the metaphase/anaphase transition. Although a portion of DdAurora keeps associated with centromeres, another portion of DdAurora protein still translocates to the central spindle at the metaphase/anaphase transition and remains there from anaphase to early cytokinesis. Another important member of the chromosomal passenger complex – DdINCENP also shows localization to the central spindle after the metaphase/anaphase transition (Chen et al., 2006). This result highly

suggests that DdAurora can form a chromosomal passenger complex with DdINCENP and translocate to the central spindle together. In the following chapter, I explored this possibility in detail.

2.3.5 DdAurora and Cytokinesis

Deletion of Aurora B by RNAi in *C.elegans* causes a defect in late cytokinesis (Schumacher et al., 1998b). Overexpression of a catalytically inactive form of Aurora B prevents the last steps of cytokinesis in mammalian cells (Terada et al., 1998). Overexpression of kinase inactive form of DdAurora in wild-type *Dictyostelium* cells also causes a cytokinesis defect. Unlike *C. elegans* cells, the contractile furrow does not initiate. This suggests that in *Dictyostelium*, DdAurora kinase activity is required in the very early stage of cytokinesis, probably as early as the actomyosin ring assembly stage.

DdINCENP begins to localize to the furrow at the very start of cytokinesis (Chen et al., 2006), while DdAurora localizes to the furrow at the very end of cytokinesis. How does DdAurora function in furrow initiation but still remain on the central spindle? This may be explained by a dynamic interaction between DdINCENP and DdAurora. DdINCENP could recruit DdAurora dynamically to the contractile furrow at the start of cytokinesis to induce furrow formation and contraction. It will be interesting to determine whether the central spindle localization of DdAurora is dynamic by photo bleaching.

Many investigators have established that the central spindle is disassembled before the end of cytokinesis and that there is no midbody structure found in *Dictyostelium* (McIntosh et al., 1985; Roos et al., 1984). However, I have shown that GFP-Aurora remains associated with the membrane of the two daughter cells at the site

of cleavage (Figure 2.3). Qian Chen made a similar observation with GFP-DdINCENP (Chen et al., 2006). Thus, it is possible that DdAurora and DdINCENP form a complex at the end of cytokinesis that may resemble a functional midbody in animal cells. This late furrow localization of the complex may be required for the final abscission of the cytoplasmic bridge connecting two daughter cells.

Chapter 3 Different Factors Regulate the Subcellular Localization of DdAurora

3.1 INTRODUCTION

Aurora kinases are highly regulated during mitosis. Different factors mediate Aurora kinase expression level, localization, activity and degradation during different stages of mitosis. Although Aurora A and Aurora B have similar expression and activity patterns during mitosis (Bischoff et al., 1998), they have distinct sub-cellular localizations and binding partners, which lead to different pathways to control their kinase activity. I will present here my studies of different factors that affect the localization and function of DdAurora.

In search of binding partners of Aurora A, TPX2 was first found to co-immunoprecipitate specifically with Aurora-A from mitotic HeLa cell extracts (Kufer et al., 2002). The N-terminus of TPX2 directly interacts with the C-terminal catalytic domain of Aurora A. TPX2 is phosphorylated by Aurora A upon binding (Kufer et al., 2002). Interestingly, the binding of TPX2 also stimulates the autophosphorylation and autoactivation of Aurora A (Eyers et al., 2003). Furthermore, phosphorylation of Aurora A can prevent phosphatase I (PPI)-induced dephosphorylation (Tsai et al., 2003). Through this positive feedback loop, the kinase activity of Aurora A is elevated by its association with TPX2. Simply put, TPX2 acts simultaneously as a binding partner, a substrate and an activator for Aurora A. In early mitosis, TPX2 is released by Ran-GTP from importin-α and importin-β (Gruss et al., 2001). Ran-GTP is generated by RCC1,

which is a GEF protein bound to chromatin (Carazo-Salas et al., 1999). Activated Aurora A then phosphorylates Eg5 to regulate spindle assembly and dynamics (Giet et al., 1999).

Aurora B has a similar binding partner – INCENP – that acts simultaneously as a substrate and activator. INCENP was first identified as the inner centromeric protein that moves from the inner centromere to the central spindle, midbody and equatorial cortex during mitosis (Cooke et al., 1987). Several other proteins were later identified with similar localization, including Aurora B (Chan and Botstein, 1993), TD-60 (Martineau-Thuillier et al., 1998), Survivin (Uren et al., 2000) and Borealin (Gassmann et al., 2004). These proteins were termed chromosomal passenger proteins based on their dynamic localization during mitosis (Earnshaw and Bernat, 1991). Together, the proteins form a chromosomal passenger complex and the complex is translocated from the centromeres to the central spindle at the metaphase/anaphase transition (Vader et al., 2006).

The interaction between INCENP and Aurora B was first reported in budding yeast (Kim et al., 1999). The interaction between these proteins is required to target them to chromosomes, central spindle and cleavage furrow (Adams et al., 2000). The highly conserved C-terminal domain of INCENP (IN-box) is responsible for its interaction with Aurora B (Honda et al., 2003). Upon binding Aurora B, INCENP is phosphorylated within the IN-box and this phosphorylation enhances the autophosphorylation and autoactivation of Aurora B kinase (Bishop and Schumacher, 2002). This positive feedback loop activation mechanism is very similar to that of Aurora A, except that Aurora A and Aurora B are activated at different localizations by different activators. Although their activation mechanisms are similar, alignment of TPX2 and INCENP did not reveal any sequence homology.

As described in the second chapter, DdAurora is the only Aurora kinase in *Dictyostelium*. Previously, I demonstrated that DdAurora localization during mitosis was similar to that of both Aurora A and Aurora B. However, the *Dictyostelium* genome does not encode for any protein similar to TPX2. In contrast, a homologue of INCENP, DdINCENP, has been shown to be a true chromosomal passenger protein that interacts with DdAurora (Chen et al., 2006). Here I explored the interaction between the two proteins in more detail.

In addition to the binding proteins described above, the function of Aurora kinases is also influenced by many other proteins. Among them, the mammalian MKLP2 protein is essential for the translocation of the chromosomal passenger complex to the central spindle (Gruneberg et al., 2004). In *Dictyostelium*, we have shown that Myosin II heavy chain is essential for proper localization of DdINCENP to the cleavage furrow (Chen et al., 2006). Here I examined whether the localization of DdAurora during mitosis was dependent on Myosin II or Kif12, a *Dictyostelium* homolog of MKLP2.

3.2 RESULTS

3.2.1 Localization of DdAurora in DdINCENP null Cells

In the previous chapter I showed that the localization of DdAurora shares characteristics of both Aurora A and Aurora B being found at the centromeres, spindle poles and central spindle at different times of mitosis. Interestingly, DdINCENP is found at some, but not all of these locations in the mitotic cell (Chen et al., 2006). Both proteins are localized on centromeres during prometaphase and metaphase and on the central

spindle during anaphase and telophase (Chen et al., 2006). In contrast, only DdAurora localizes to the spindle poles in prometaphase and metaphase and leaves the poles in anaphase (Figure 2.4), whereas DdINCENP localizes at the spindle poles only during anaphase and telophase (Chen et al., 2006). Finally, DdAurora, but not DdINCENP remains associated with centromeres after the metaphase/anaphase transition (Figure 2.5). The similarities and differences in localization of these two proteins suggest that they may associate to each other only at specific locations. Thus, I hypothesized that the localization of DdAurora may be dependent on DdINCENP only at the places where they colocalize. To test this hypothesis and to determine whether DdAurora forms a chromosomal passenger complex with DdINCENP at the centromeres and central spindle, I examined endogenous DdAurora localization in DdINCENP null cells expressing the centromeric marker HcpA-GFP (Kaller et al., 2006).

I found that the localization of DdAurora on the spindle poles of metaphase cells was not influenced by the loss of DdINCENP (Figure 3.1a). This observation may suggest that the polar localization of DdAurora is analogous to that of Aurora A and therefore not affected by DdINCENP. In contrast, DdAurora failed to localize to the central spindle in the absence of DdINCENP and was absent from the central spindle during anaphase and telophase (Figure 3.1b,c). This finding suggests that DdINCENP is required for DdAurora localization to the central spindle at the metaphase/anaphase transition. This result also suggests that DdINCENP may be an essential binding partner of DdAurora.

Surprisingly, DdAurora still localized to the centromeres of metaphase cells and colocalized with HcpA-GFP extensively at the metaphase plate even in the absence of

DdINCENP (Figure 3.1a). This observation suggests that DdINCENP is not required for the localization of DdAurora to centromeres. I suggest that DdAurora may interact with other factors in order to associate with centromeres in *Dictyostelium*, which would explain its localization to the centromeres throughout the majority of M phase. Furthermore, DdAurora kept associated with centromeres during anaphase and telophase (Figure 3.1b,c). Remarkably, in the absence of DdINCENP, HcpA was no longer displaced from the centromeres at the metaphase/anaphase transition and it colocalized with DdAurora at the centromeres (Compare Figure 2.5b with Figure 3.1b). Since DdINCENP is a potential partner and activator of DdAurora, it is possible that the loss of DdINCENP renders the centromeric DdAurora inactive and unable to displace HcpA from the centromere at the metaphase/anaphase transition. DdINCENP null cells have been shown to have some defects in chromosome segregation (Chen et al., 2006), these defects may be caused by improper displacement of HcpA from the centromeres at the metaphase/anaphase transition.

In the absence of DdINCENP, the contractile furrow localization of DdAurora in late cytokinesis was also lost (data not shown). This suggests that DdINCENP is also required for DdAurora localization to the cleavage furrow. The central spindle localization of DdAurora in anaphase and telophase may be a requirement for its subsequent localization at the cleavage furrow. The absence of DdAurora at the cleavage furrow may be the cause of the cytokinesis defect observed in DdINCENP null cells (Chen et al., 2006).

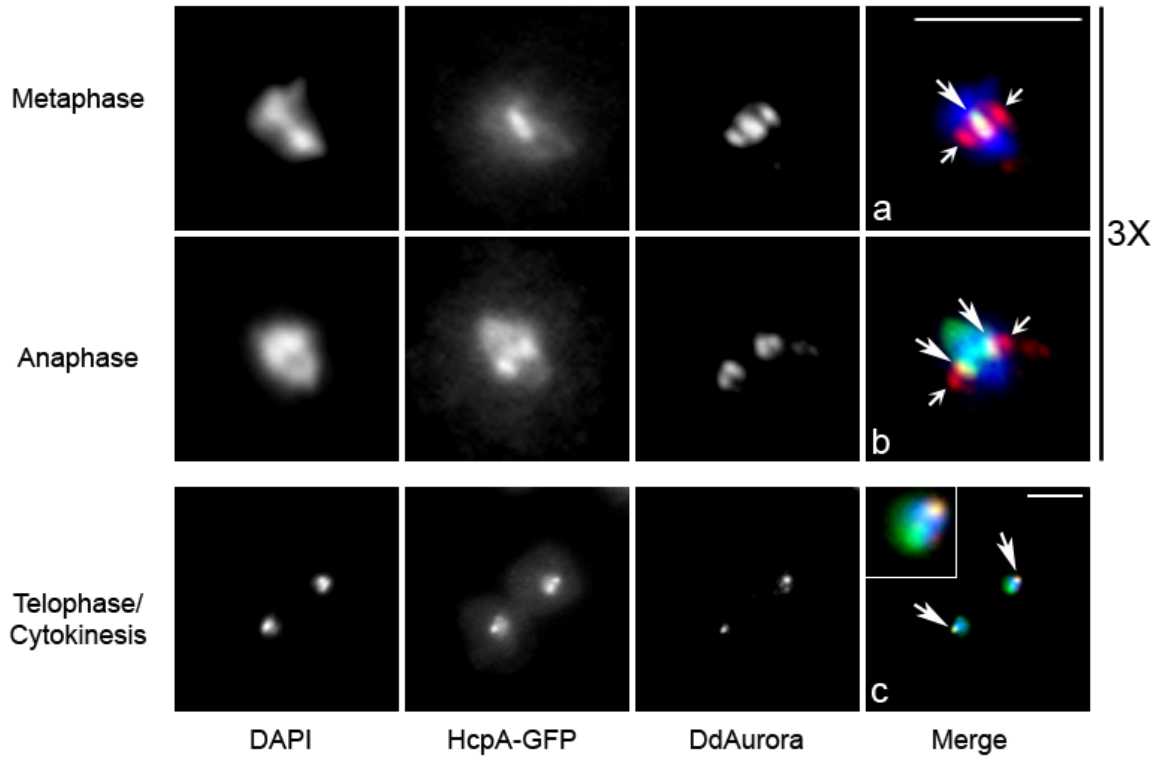

Figure 3.1 DdINCENP is essential for DdAurora localization to the central spindle but not the centromeres.

Localization of DdAurora and HcpA-GFP during different stages of mitosis in DdINCENP null cells was examined by immunofluorescence microscopy. In the merged images, DNA is shown in blue, endogenous DdAurora is shown in red and HcpA-GFP is shown in green. (a) In metaphase, DdAurora localized to the centromeres (shown by arrows) and spindle poles (shown by small arrows). Centromeric DdAurora colocalized with HcpA-GFP extensively. (b) In early anaphase, DdAurora localized to the spindle poles (small arrows) and centromeres (arrows) but not the central spindle. Surprisingly, HcpA still associated with the centromeres and colocalized with centromeric DdAurora (arrows) in anaphase in DdINCENP null cells. In wild-type cells, HcpA displayed a diffuse localization in anaphase (Figure 2.5). (c) In late telophase/cytokinesis, DdAurora was still associated with centromeres and colocalized with HcpA-GFP (arrows). Bar, 5μm. The top panel is enlarged 3x magnification.

3.2.2 DdAurora Forms a Chromosomal Passenger Complex with DdINCENP

To explore the relationship between DdAurora and DdINCENP in more detail, I studied DdINCENP null cells expressing GFP-DdINCENP. The cytokinesis defect of DdINCENP null cells is fully rescued by exogenous expression of GFP-DdINCENP (Chen et al., 2006). I determined the localization of endogenous DdAurora by immunofluorescence studies with this cell line.

Figure 3.2 shows that GFP-INCENP rescued the translocation of DdAurora to the central spindle at the metaphase/anaphase transition and extensively colocalized with DdAurora at the central spindle. This result strongly suggested that DdINCENP and DdAurora formed a chromosomal passenger complex that translocated from the centromeres to the central spindle at the metaphase/anaphase transition.

To determine whether DdAurora can truly form a complex with DdINCENP, pull-down experiments with TAP-tagged GFP-DdAurora were performed. Blotting of the bound fractions with DdINCENP antibodies revealed that endogenous DdINCENP was co-purified with TAP-GFP-DdAurora but not with TAP-GFP alone (Figure 3.3A). This finding demonstrated that DdAurora forms a complex with DdINCENP *in vivo*. The interaction between these two proteins was further confirmed by immunoprecipitation of endogenous DdAurora from the GFP-DdINCENP rescued DdINCENP null cell line. I found that GFP-DdINCENP was co-immunoprecipitated with endogenous DdAurora (Figure 3.3B). This result confirmed the direct interaction between DdAurora and DdINCENP *in vivo*.

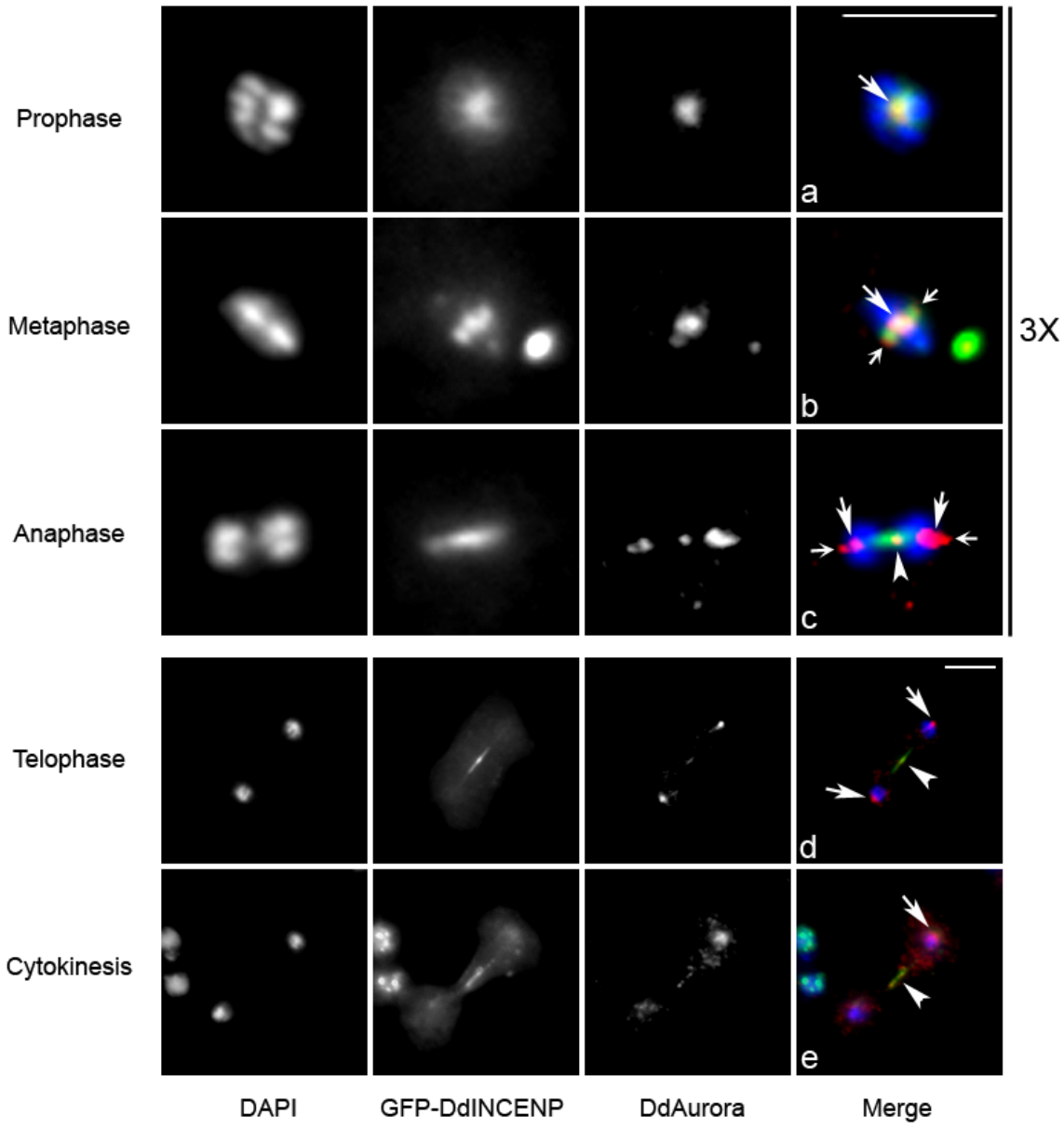

Figure 3.2 GFP-DdINCENP rescues the translocation of DdAurora to the central spindle in DdINCENP null cells.

GFP-DdINCENP rescued DdINCENP null cells were immunostained with anti-DdAurora antibodies. In the merged images, DNA is shown in blue, GFP-DdINCENP is shown in green and endogenous DdAurora is shown in red. (a) In prophase, DdAurora and DdINCENP colocalize at the centromeres (shown by arrow). (b) In metaphase, DdAurora clearly showed colocalization with GFP-DdINCENP at the centromeres (shown by arrow). (c) In early anaphase, a portion of DdAurora translocated from centromeres to the central spindle. GFP-DdINCENP showed the same translocation at the metaphase/anaphase transition and colocalized with DdAurora at this location (shown by arrowhead). (d,e) From telophase to early cytokinesis, DdAurora colocalized with GFP-DdINCENP at the central spindle (shown by arrowheads) from. Bar, 5μm. The top panel is enlarged 3x magnification.

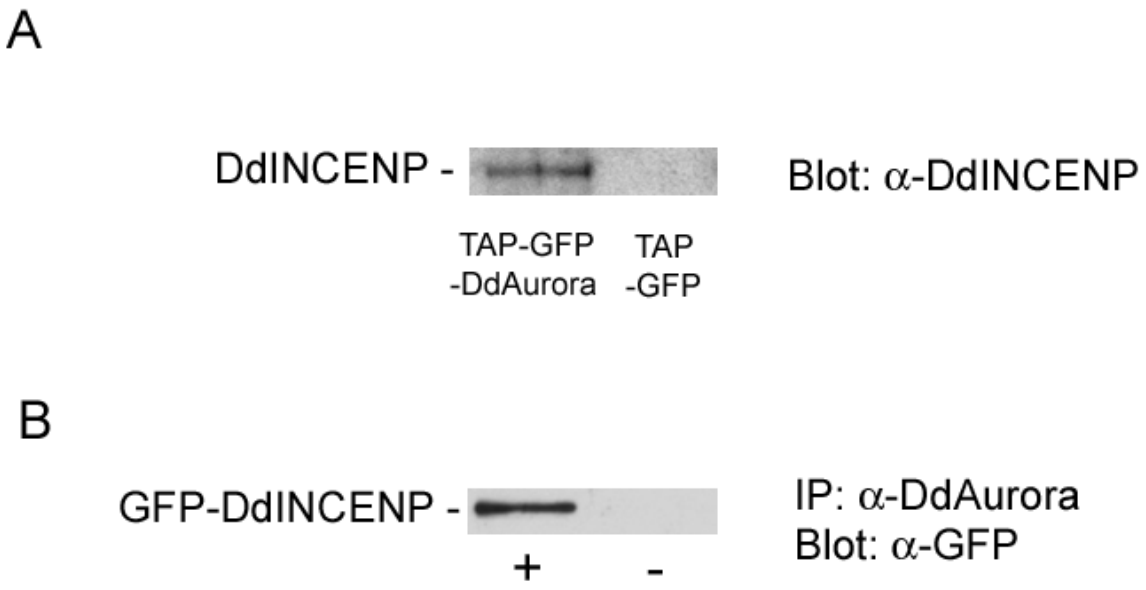

Figure 3.3 DdAurora interacts with DdINCENP *in vivo*.

(A) TAP-GFP-DdAurora was purified from wild-type cells. Endogenous DdINCENP was co-purified with TAP-GFP-DdAurora but not with a TAP-GFP control. The TAP-GFP fusion protein was used as a negative control. The elutions were subjected to western blotting with anti-DdINCENP antibodies.

(B) In GFP-DdINCENP rescued DdINCENP null cells, endogenous DdAurora was immunoprecipitated by affinity purified DdAurora antibodies. Elutions were examined with anti-GFP antibodies. GFP-DdINCENP was found co-immunoprecipitated with DdAurora but not in the no-antibody control.

3.2.3 DdINCENP does not Require DdAurora to Localize at the Central Spindle

The conserved C-terminal IN-box domain of INCENP is known to be essential for its interaction with Aurora B and activation of the kinase (Bishop and Schumacher, 2002; Honda et al., 2003). DdINCENP also has the highly conserved IN-box domain at its C-terminus. To determine the functional contribution of different DdINCENP domains, a series of DdINCENP truncation mutants were constructed by Qian Chen, a student in our laboratory (Chen et al., 2007). The GFP-tagged truncation mutants were transformed into DdINCENP null cells to study their localization patterns during mitosis. Qian Chen demonstrated that the N-terminal domain of DdINCENP was necessary and sufficient to localize to the cleavage furrow. A construct containing this domain (DdINCENP-ΔC, Figure 3.4A) localized to the cleavage furrow, while one lacking this domain (DdINCENP-ΔN, Figure 3.4A) did not. Interestingly, both constructs were able to localize to the central spindle in anaphase (Chen et al., 2007). The observation that DdINCENP-ΔC localized on the central spindle could be interpreted in two ways. Since this protein is lacking the IN-box domain assumed to be necessary for the interaction with DdAurora, DdINCENP-ΔC may localize on the central spindle in a DdAurora-independent manner. Alternatively, it is possible that DdINCENP-ΔC can still interact with DdAurora by a domain other than the IN-box domain. To discern among these possibilities, I investigated the interactions between different DdINCENP truncation mutants and DdAurora and their influence on the localization of endogenous DdAurora.

I determined the localization of endogenous DdAurora in DdINCENP null cells expressing the two truncated DdINCENP proteins (Figure 3.4B). DdAurora translocated to the central spindle of cells expressing DdINCENP-ΔN, similar to the localization pattern of DdAurora in cells expressing full-length GFP-DdINCENP. However, in GFP-DdINCENP-ΔC expressing cells, the localization of DdAurora at the central spindle was completely lost. In all cases, DdAurora localized normally to the spindle poles in metaphase (data not shown), and to the centromeres of the separated chromosomes in telophase (Figure 3.4).

These results suggested that the localization of DdINCENP at the central spindle does not depend on its interaction with DdAurora. In addition, they indicate that the C-terminal IN-box domain of DdINCENP is critical for its interaction with DdAurora and is required for DdAurora translocation to the central spindle.

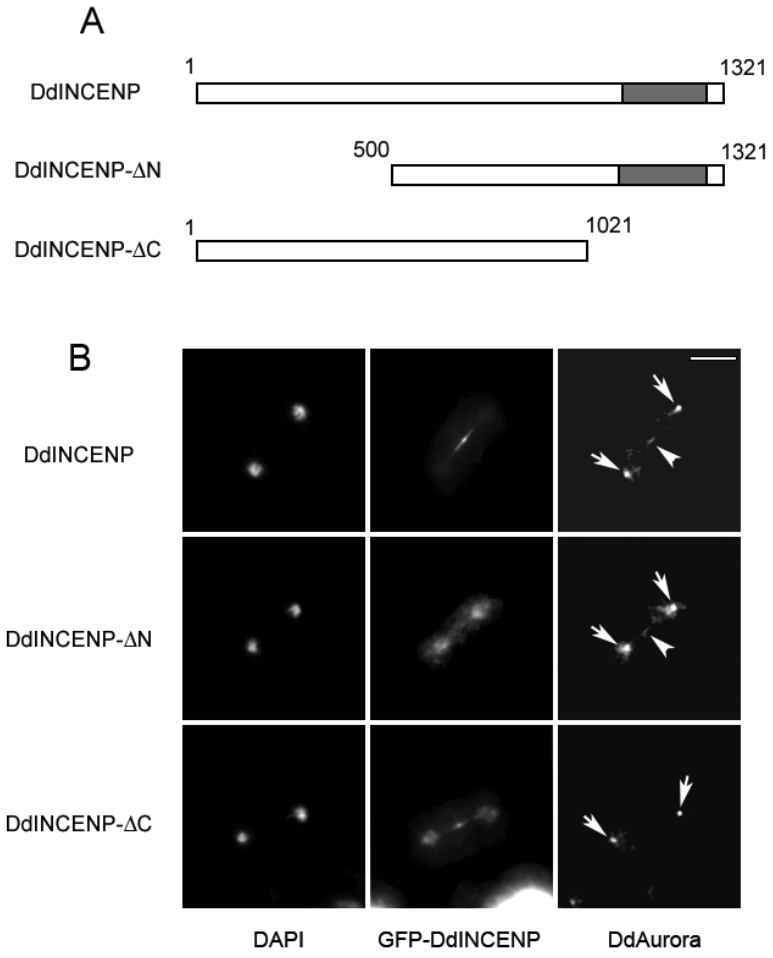

Figure 3.4 Various truncation mutants of DdINCENP translocate DdAurora to the central spindle differently.

(A) Schematic diagram of different truncation mutants of DdINCENP. The conserved IN-box domain, which is essential for Aurora B binding and activation is shown in gray.

(B) Differently truncated GFP-DdINCENPs were introduced into DdINCENP null cells and the central spindle localization of endogenous DdAurora (shown by arrowheads) was examined by immunofluorescence microscopy. The central spindle localization of DdAurora was rescued in GFP-DdINCENP and GFP-DdINCENP-ΔN tramsformed cells (shown by arrowheads), but not in GFP-DdINCENP-ΔC transformed cells. Cells in telophase, which can display the central spindle localization of DdAurora most clearly, were shown in these images. Bar, 5μm.

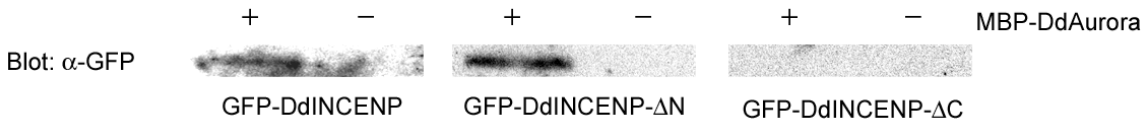

Figure 3.5 Different truncations of DdINCENP interact with DdAurora differently.

In vitro pull-down assay was used to determine the interaction between maltose binding protein (MBP)-DdAurora and GFP-DdINCENP truncations expressed in DdINCENP null cells. Purified MBP-Aurora bound amylose beads were used for this assay. Cell extracts from DdINCENP null cells expressing individual truncation mutants were mixed with the beads to examine the interaction between MBP-DdAurora and the truncation mutants. Blotting of the bound fractions with anti-GFP antibodies showed that GFP-DdINCENP and GFP-DdINCENP-ΔN could be pulled down with MBP-DdAurora, but GFP-DdINCENP-ΔC could not. Amylose beads without MBP-Aurora bound were used as negative controls.

To test whether the localization results described above are mediated by the interaction between various DdINCENP truncations and DdAurora, I performed pull-down experiments on all different cell lines with recombinant DdAurora protein. Cell extracts from DdINCENP null cells expressing individual truncation mutants were incubated with MBP (Maltose-binding protein)-DdAurora bound to amylose beads. I found that the DdINCENP construct that still contains the IN-box domain (DdINCENP-ΔN) interacted with DdAurora and co-precipitated with the MPB-DdAurora beads (Figure 3.5). In contrast, the truncated form of DdINCENP lacking the IN-box domain (DdINCENP-ΔC) could not be pulled down with MBP-DdAurora.

Based on these results, I concluded that the interaction between DdINCENP and DdAurora is essential for DdAurora's translocation to the central spindle at the metaphase/anaphase transition. Specifically, the C-terminal IN-box domain is critical for the interaction between DdINCENP and DdAurora.

3.2.4 The Kinesin-related Protein Kif12 is Required for the Localization of DdAurora at the Central Spindle

In mammalian cells, the kinesin-like protein MKLP2 is essential for the localization of the chromosomal passenger complex to the central spindle (Gruneberg et al., 2004). In *Dictyostelium*, Kif12 was identified as a kinesin-like protein required for myosin localization to the furrow. Kif12 null cells have severe cytokinesis defects (Lakshmikanth et al., 2004). This phenotype suggested that *Dictyostelium* Kif12 may play a similar role to metazoan MKLP2 and may facilitate the redistribution of the

chromosomal passenger complex from the centromeres to the central spindle at the metaphase/anaphase transition.

To test whether Kif12 acts in this capacity, Kif12 null cells expressing HcpA-GFP were immunostained with anti-DdAurora antibodies to determine the localization of endogenous DdAurora in these cells. Similar to my observations of DdINCENP null cells, the central spindle localization of DdAurora was completely lost from anaphase to telophase in Kif12 null cells (Figure 3.6b,c). Furthermore, while DdAurora did not localize to the central spindle in Kif12 null cells, it still localized to the centromeres throughout mitosis and colocalized extensively with HcpA-GFP in metaphase and late telophase (Figure 3.6a,c). Again, similarly to what I found in wild-type cells (Figure 2.5b), HcpA-GFP was diffusely distributed in the nucleus of Kif12 null cells during early anaphase (Figure 3.6b). This finding suggests that although Kif12 mediates the translocation of the DdAurora/DdINCENP complex to the central spindle, it might not be required for the activation of DdAurora at the centromeres in metaphase/anaphase. In the absence of Kif12, DdAurora may still be activated by DdINCENP displacing HcpA from the anaphase centromeres.

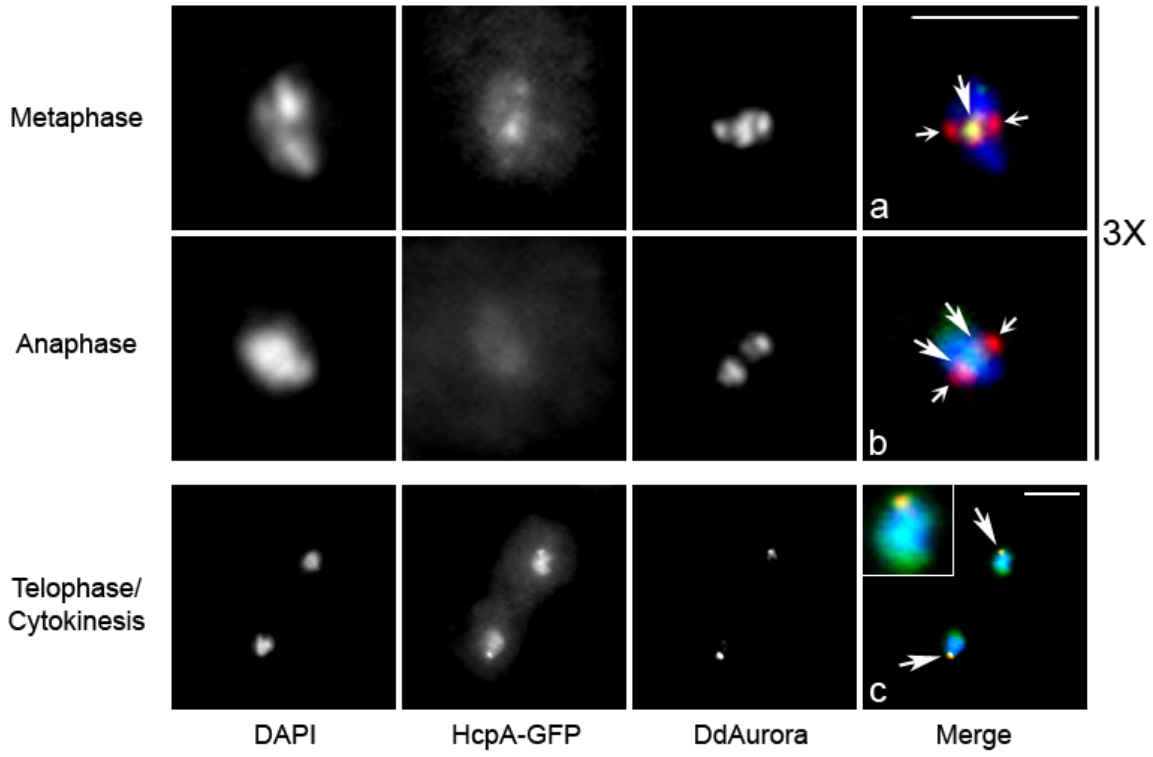

Figure 3.6 Kif12 is essential for the central spindle localization of DdAurora but not the centromeric localization.

Localization of endogenous DdAurora in Kif12 null cells was determined by immunofluorescence microscopy. In the merged images, DNA is shown in blue, DdAurora is shown in red and HcpA-GFP is shown in green. (a) In metaphase, DdAurora localized to the centromeres (shown by arrows) and spindle poles (shown by small arrows). Centromeric DdAurora colocalized with HcpA-GFP extensively. (b) In early anaphase, DdAurora localized to the spindle poles (small arrows) and centromeres (arrows) but not the central spindle. Interestingly, HcpA showed diffused localization in anaphase in Kif12 null cells, similar to HcpA-GFP localization in wild-type cells at this stage (Figure 2.5). (c) In late telophase/cytokinesis, DdAurora kept associated with centromeres and colocalized with HcpA-GFP (arrows). Bar, 5μm. The top panel is enlarged 3x magnification.

This finding suggests that while Kif12 is not required for the centromeric localization of DdAurora, Kif12 is necessary for the localization of DdAurora at the central spindle. Although Kif12 may be involved in the movement of DdAurora and the chromosomal passenger complex during mitosis, I could not demonstrate a physical interaction between these two proteins. Immunoprecipitation of Kif12 null cells expressing GFP-Kif12 with anti-DdAurora antibodies failed to reveal a direct interaction between GFP-Kif12 and DdAurora (data not shown). It is possible that the two proteins associate indirectly or that their interaction is highly dynamic and cell-cycle-regulated.

3.2.5 Overexpression of GFP-DdINCENP Rescues DdAurora Localization at the Central Spindle in Kif12 null Cells

While I found that DdAurora is absent from the central spindle of Kif12 null cells, Qian Chen found that GFP-DdINCENP is localized at the central spindle in these mutant cells (Chen et al., 2007). These divergent observations could be explained by two possible models. One is that DdINCENP does not require DdAurora to localize at the central spindle. In favor of this model I have shown that a truncated form of DdINCENP lacking the IN-box domain (DdINCENP-ΔC) localized at the central spindle even though DdAurora was not there (Figure 3.4). A consequence of this model is that Kif12 is important for the localization of DdAurora, but not DdINCENP, at the central spindle. Alternatively, it is possible that the overexpression of GFP-DdINCENP in Kif12 null cells (these cells still contain endogenous levels of untagged DdINCENP) rescues the localization of both chromosomal passenger proteins at the central spindle. To resolve this issue I studied the phenotype of Kif12 null cells expressing GFP-DdINCENP.

Immunostaining of these cells revealed that the overexpression of GFP-DdINCENP restored the localization of DdAurora at the central spindle in anaphase and telophase (Figure 3.7A). Furthermore, immunoprecipitation assays showed that GFP-DdINCENP interacted with DdAurora in the absence of Kif12 (Figure 3.7B). These results strengthen the model that DdAurora and DdINCENP form a complex and localize together at the central spindle. I would also suggest that Kif12 is required for the localization of endogenous DdINCENP/DdAurora complex at the central spindle. Unfortunately, the available DdINCENP antibodies are not of enough quality for immunofluorescence studies of Kif12 null cells.

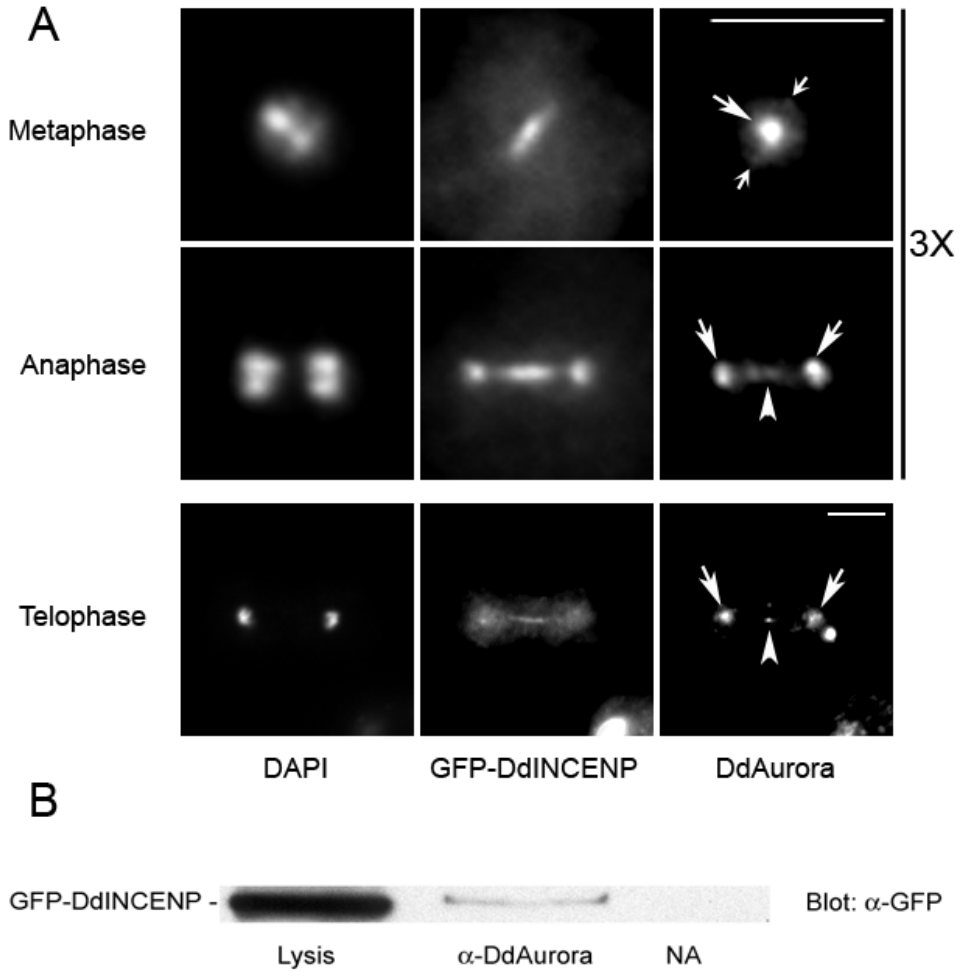

Figure 3.7 Overexpression of GFP-DdINCENP in Kif12 null cells rescues localization of DdAurora to the central spindle.

(A) Co-localization of DdAurora and GFP-DdINCENP in Kif12 null cells. In metaphase, DdAurora localized at the spindle poles (small arrows) and centromeres localized at the metaphase plate (shown by arrow). In anaphase and telophase, the central spindle localization (shown by arrowheads) of DdAurora was rescued by the overexpression of GFP-DdINCENP. Bar, 5μm.

(B) GFP-DdINCENP was co-immunoprecipitated with DdAurora in the absence of Kif12. Affinity purified anti-DdAurora antibodies were used to perform the immunoprecipitation assay. The bound fractions were analyzed with anti-GFP antibodies. Immunoprecipitation without antibodies was used as a negative control.

Although GFP-DdINCENP still localizes to the central spindle in Kif12 null cells, the cleavage furrow localization of GFP-DdINCENP is disrupted in these cells (Chen et al., 2007). Because the late furrow localization of endogenous DdAurora is difficult to observe with immunostaining, the requirement of Kif12 for the localization of DdAurora to the furrow is still unknown. Since DdINCENP is required for the localization of DdAurora to the central spindle, it may also be required for the furrow localization of DdAurora in cytokinesis. I predict that the localization of DdAurora to the cleavage furrow may be disrupted in Kif12 null cells.

3.2.6 Myosin II is Required for the Cleavage Furrow Localization of DdAurora

We have shown previously that the distribution of DdINCENP at the cleavage furrow was abnormal in Myosin II mutants. In these cells GFP-DdINCENP localized to a narrow band at the furrow instead of the broad cleavage furrow cortex localization observed in wild type cells (Chen et al., 2006). To determine the effect of Myosin II on DdAurora localization, I immunostained Myosin II heavy chain null cells with affinity purified DdAurora antibodies. Immunostaining images showed that the translocation of endogenous DdAurora to the central spindle was normal in these mutant cells. In addition, the centromeric localization of DdAurora in metaphase was not affected (Figure 3.8A). To examine the localization of DdAurora in Myosin II null cells during late cytokinesis, I expressed GFP-DdAurora in these cells. I showed previously that GFP-DdAurora labels the cytoplasmic bridge connecting wild type cells near the end of cytokinesis (Figure 2.3h). Cells undergoing cytokinesis were examined by live-cell fluorescence microscopy. Figure 3.8B shows that GFP-DdAurora localized normally to

the central spindle (arrowhead), but was absent from the cytoplasmic bridge (arrow) during late cytokinesis. This defect may be caused by the abnormal localization of DdINCENP at the cleavage furrow in cytokinesis.

In conclusion, Myosin II is not required for the central spindle translocation of DdAurora at the metaphase/anaphase transition, but it is important for the localization of the chromosomal passenger complex to the cleavage furrow during cytokinesis.

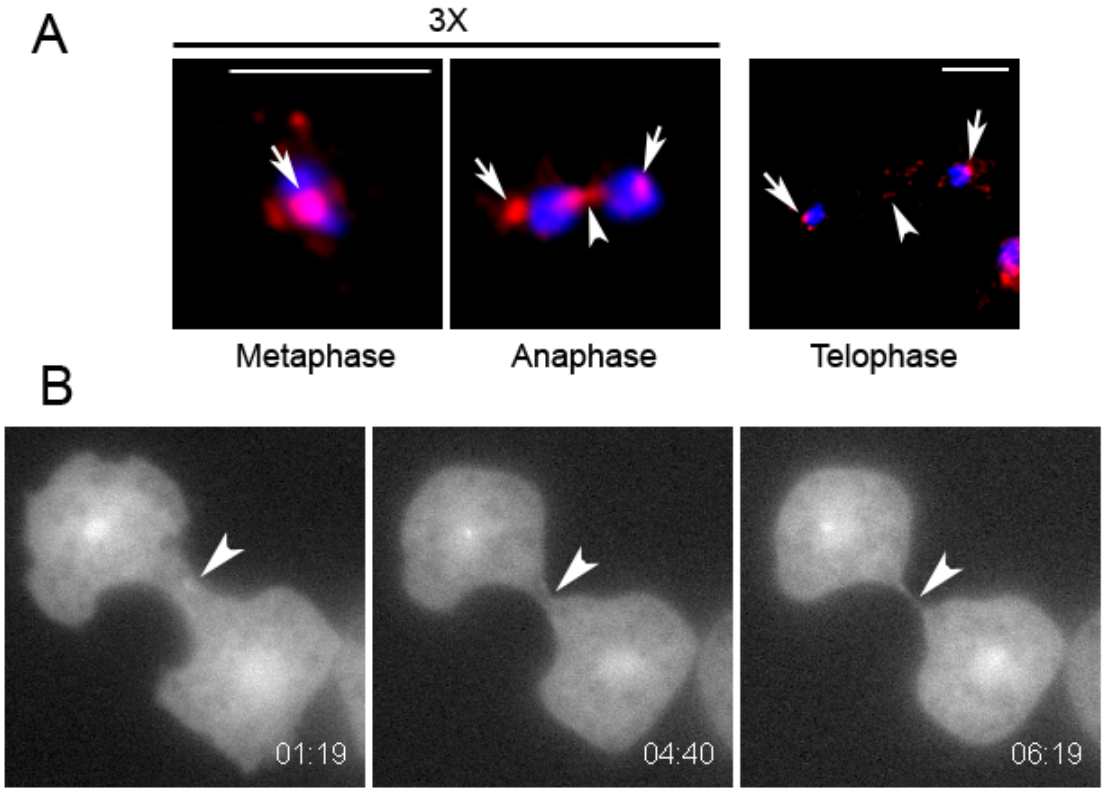

Figure 3.8 Localization of GFP-DdAurora in Myosin II null cells.

(A) Localization of endogenous DdAurora in Myosin II null cells was determined by immunostaining. In the merged images, DNA is shown in blue and endogenous DdAurora is shown in red. The central spindle localization of DdAurora (shown by arrowheads) in both anaphase and telophase was normal in these cells. The centromeric localization of DdAurora was not affected either (shown by arrows). Bar, 5μm. The left panel is enlarged 3x magnification.

(B) Live images of GFP-Aurora transformed Myosin II null cells undergoing cytokinesis. The central spindle localization of GFP-DdAurora could still be observed at 01:19 (shown by arrowhead), but the late furrow localization was missing (shown by arrowheads) compared to the intense late furrow localization of GFP-DdAurora in wild-type cells (Figure 2.3). Bar, 5μm.

3.3 DISCUSSION

3.3.1 DdAurora and DdINCENP

In Chapter 2, I showed that DdAurora shares sequence and localization similarities with both Aurora A and Aurora B. In this chapter I further demonstrated that DdAurora can form a chromosomal passenger complex with DdINCENP, by both immunoprecipitation and dependence for central spindle localization. Consistent with findings in other organisms, the IN-box domain of DdINCENP is essential for the binding to DdAurora and the localization of DdAurora to the central spindle. However, the spindle pole localization of DdAurora does not involve an interaction with DdINCENP. These results suggest that the Aurora A-like function of DdAurora may be mediated by some other factors. Furthermore, the centromeric localization of DdAurora is not affected by the absence of DdINCENP either. This phenomenon has not been reported in other species. This finding suggests that DdAurora may have some additional functions not yet recognized in other Aurora kinases, which makes it a special member of this kinase family.

As a member of the chromosomal passenger complex, DdAurora localizes to the centromeres, central spindle and cleavage furrow in different stages of mitosis and cytokinesis. However, unlike animal Aurora B, DdAurora does not always colocalize with DdINCENP at these localizations. At the metaphase/anaphase transition, GFP-DdINCENP translocates completely from the centromeres to the central spindle while a large portion of DdAurora keeps associated with the centromeres. When the cleavage

furrow starts to form in telophase, GFP-DdINCENP translocates to the cortex region of the furrow. However DdAurora can only be detected on the furrow at a very late stage of cytokinesis. It is possible that, instead of forming a stable complex, DdINCENP interacts with DdAurora dynamically. DdINCENP most likely works as a scaffolding protein of the chromosomal passenger complex and recruits other members of the complex to its periphery to execute their functions. Since INCENP also acts as the activator of Aurora B, this dynamic binding and activation mechanism may be used to finely control the activity of Aurora at a particular location.

The differences in DdAurora and DdINCENP localization also suggest that, instead of being solely a DdAurora co-factor, DdINCENP may play additional roles in mitosis and cytokinesis by interacting with other proteins. For example, it has been shown recently that phosphorylated INCENP can bind to Polo-like kinase (PLK) (Goto et al., 2006), another important mitotic kinase essential for mitotic entry, spindle formation and cytokinesis (van de Weerdt and Medema, 2006). DdINCENP may be able to regulate mitosis through the interaction with the PLK protein in *Dictyostelium*.

It has been shown that mammalian HP1 is displaced from mitotic chromosomes when Aurora B phosphorylates histone H3 in M phase (Fischle et al., 2005; Hirota et al., 2005). As a hallmark of mitosis, HP1 displacement may be important for mediating proper chromatin condensation and faithful chromosome segregation during mitosis (Fischle et al., 2005). In my research, it appears that DdINCENP and DdAurora are also important to regulate the association of HcpA-GFP, a *Dictyostelium* homolog of HP1 (Kaller et al., 2006), with centromeres. I showed that HcpA-GFP was displaced from wild-type centromeres during early anaphase but that it remained on the centromeres of

DdINCENP null cells. DdAurora was clearly present on centromeres in both metaphase and anaphase of wild type and mutant cells (Figure 3.1). However, in the absence of DdINCENP, DdAurora may no longer be activated at the centromeres and thus may be unable to displace HcpA from those centromeres. Failure to displace HcpA from centromeres may, in turn, may cause the chromosome segregation defects observed in DdINCENP null cells (Chen et al., 2006).

3.3.2 DdAurora and Kif12

Similarly to the role of the animal motor protein MKLP2, *Dictyostelium* Kif12 is essential for the localization of DdAurora to the central spindle at the metaphase/anaphase transition. It is likely that Kif12 is also important for the localization of DdINCENP to the central spindle. However, when GFP-DdINCENP is overexpressed in Kif12 null cells, it is able to localize to the central spindle and rescues the localization of DdAurora at this site. The localization of GFP-DdINCENP at the central spindle may be explained by the microtubule binding ability of DdINCENP. In the absence of Kif12, overexpression of GFP-DdINCENP may improve its ability to bind to the central antiparallel microtubule arrays through its affinity to microtubules. In this way it may be able to then recruit DdAurora and other proteins to the central spindle and help alleviate the cytokinesis defect of Kif12 null cells (Qian Chen, personal communication).

Additionally, it appears that Kif12 is not required for the activation of DdAurora at the centromeres. As discussed above, DdAurora activity may be required for the displacement of HcpA from centromeres at the metaphase/anaphase transition. In Kif12 null cells HcpA-GFP is normally displaced from centromeres in early anaphase, similar

to what was observed in wild-type cells. This finding suggests that DdAurora activity at the centromeres is not affected by the loss of Kif12.

3.3.3 DdAurora and Myosin II

Qian Chen has shown that DdINCENP interacts with the actin cytoskeleton by a domain found at its N-terminus (Chen et al., 2007). This interaction is important to localize DdINCENP at the cleavage furrow. In addition, the organization of the cytoskeleton at the cleavage furrow is also important for the correct distribution of DdINCENP at this location (Chen et al., 2006). DdINCENP is still localized at the cleavage furrow of Myosin II null cells but its distribution is abnormal (Chen et al., 2006).

I demonstrated here that the localization of DdAurora was also affected by the absence of Myosin II. Although GFP-DdAurora was still localized at the central spindle it was absent from the cleavage furrows of myosin mutant cells. This suggests that the absence of Myosin II not only affects localization of DdINCENP at the contractile furrow, but also disrupts the furrow localization of DdAurora. Although Myosin II is the motor protein driving the contractile ring, it has not been shown that it is responsible for the transportation of the chromosomal passenger complex from the central spindle to the cleavage furrow. The distorted localization of the DdAurora/DdINCENP complex to the cleavage furrow may be explained by a disordered actomyosin ring and cytoskeletal structure at the cleavage furrow in the absence of Myosin II heavy chain.

Finally, since Aurora kinase activity is important for the completion of cytokinesis, the cytokinesis defect of Myosin II null cells could be explained by both

failure of contraction and the absence of Aurora kinase activity at the furrow during late cytokinesis.

Chapter 4 Potential Binding Partners of DdAurora

4.1 INTRODUCTION

In previous chapters I have shown that DdAurora can interact with DdINCENP to function at the central spindle and cleavage furrow. How DdAurora localizes to the spindle poles and the centromeres is not clear. TPX2 was identified as a binding partner of Aurora A and is required for spindle localization and activation of Aurora A (Eyers et al., 2003; Kufer et al., 2002). Searching the database for a TPX2 homolog in *Dictyostelium* failed to reveal any related protein. How is then DdAurora localized to spindle poles and activated in early mitosis? Since DdINCENP is larger than other INCENP proteins and only their C-terminus IN-box domains are conserved, is it possible that DdINCENP has dual roles of TPX2 and INCENP? Alignment of DdINCENP and TPX2 failed to show sequence homology between their N-terminal domains. Since GFP-DdINCENP-ΔC did not show an interaction with DdAurora in previous co-immunoprecipitation test, I ruled out this possibility (Chapter 3.2.3). Thus, the binding partner of DdAurora at spindle poles is still unknown.

Although certain substrates of Aurora kinases have been identified in different stages of mitosis, such as TACC and Eg5 in prometaphase (Giet et al., 2002; Giet et al., 1999), CENP-A and H3 in metaphase (Giet and Glover, 2001; Kunitoku et al., 2003), and MgcRacGAP in cytokinesis (Minoshima et al., 2003), it is still unclear how Aurora kinases function in different stages of mitosis. Identification of novel binding partners of

Aurora kinases by biochemical approaches may be an efficient way to elucidate the function of these complex kinases.

With the exception of DdINCENP, no other binding partner or substrate of DdAurora has been identified in *Dictyostelium*. Because DdAurora combines many characteristics of both Aurora A and Aurora B, I attempted to identify novel binding partners or substrates of DdAurora by large-scale immunoprecipitation.

4.2 RESULTS

Using affinity purified polyclonal antibodies against DdAurora, I was able to perform large-scale immunoprecipitations to pull down potential binding partners of endogenous DdAurora in wild type cells. Cell extracts from wild-type AX2 cells were mixed with affinity purified anti-DdAurora antibodies and the antibody-DdAurora complexes were precipitated with protein-A beads. The bound fractions were then resolved on a polyacrylamide gel. As a negative control I performed parallel precipitations using an irrelevant Rabbit IgG. Compared to the control precipitation, the DdAurora immunoprecipitation contained four proteins that were copurified specifically with endogenous DdAurora. These proteins had relative molecular weights of 240kD, 180kD, 150kD and 60kD respectively (Figure 4.1). These bands were cut out from the gel and subjected to mass spectrometry analysis following in-gel digestion. All four bands were successfully identified as proteins found in the *Dictyostelium* genome database.

The first candidate migrating at 240kD was identified as pyr1-3 (entry DDB0201646 in http://dictybase.org; Genebank accession XP_643196), a

multifunctional enzyme that carries out the three first enzymatic activities of the *de novo* pyrimidine biosynthetic pathway. Since this protein has not been show to have any connection to mitosis and the same protein was independently copurified with TAP-tagged LvsB (Elena Kypri, personal communication), a protein involved in lysosome regulation (Kypri et al., 2007), pyr1-3 was considered as a nonspecific background product of the purification. The second and the third bands migrating at 180kD and 150kD were both identified as *Dictyostelium* topoisomerase B (entry DDB0231510 in http://dictybase.org; Genebank accession XP_646786). The lower 150kD band could be a degradation product of the full-length topoisomerase B protein. Topoisomerase B is the *Dictyostelium* homolog of topoisomerase II, which has been shown to be essential for chromosome condensation and segregation (Uemura et al., 1987). Interestingly, topoisomerase II has been identified as a potential substrate of Aurora B by a proteomic approach (Morrison et al., 2002). Topoisomerase B could be an interesting potential substrate of DdAurora in *Dictyostelium*. The fourth band migrating at 60kD was identified as *Dictyostelium* Hsp60 (HspA) (entry DDB0219929 in http://dictybase.org; Genebank accession XP_636839). Because of the chaperone nature of this heat shock protein, it could be either a real partner of DdAurora or a chaperone of nascent DdAurora.

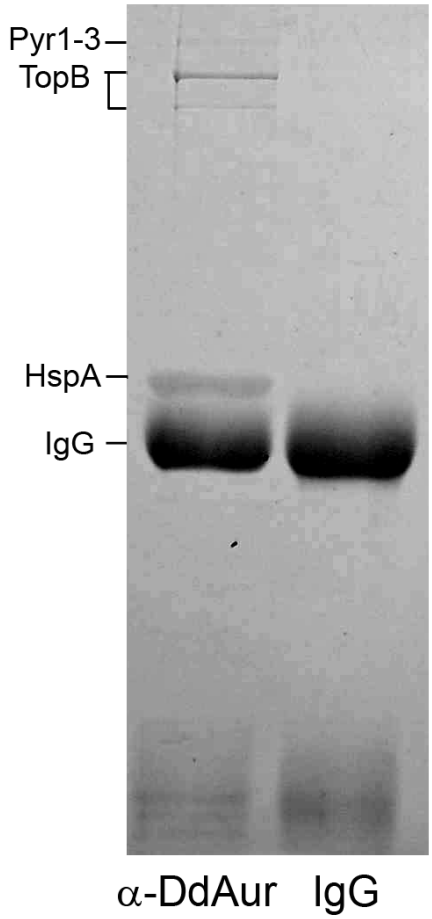

Figure 4.1 Purification of potential binding partners of DdAurora by large-scale co-immunoprecipitation.

Endogenous DdAurora was purified by large-scale immunoprecipitation with affinity purified anti-DdAurora antibodies. Unspecific Rabbit IgG was used as a negative control. Final purification fractions were resolved on a 10% polyacrylamide gel and stained by coomassie blue. Comparing to the negative control, four potential binding partner bands were observed in the anti-DdAurora lane, which were migrating at 240kD, 180kD, 150kD and 60kD respectively. These bands were cut out from the gel and subjected to mass spectrometry following in-gel digestion. They were all identified as corresponding proteins in *Dictyostelium*. The top band migrating at 240kD was identified as pyr1-3. The second and the third band were both identified as topoisomerase B, the lower 150kD band could be a degradation product of full-length topoisomerase B. The band right above the IgG band was identified as HspA, a homolog of Hsp60 in *Dictyostelium*. The DdAurora band co-migrates with the IgG band, so it cannot be seen on this gel.

DdAurora co-migrates with the large IgG band, so it is difficult to be resolved from the gel. DdINCENP was also co-immunoprecipitated with DdAurora, but because it is degraded to a very low level, it is detectable only by western blot (data not shown).

4.3 DISCUSSION

4.3.1 TopB is a Potential Substrate of DdAurora

Topoisomerase II has been identified previously as a potential substrate of Aurora B by a proteomic approach (Morrison et al., 2002). Topoisomerase II extracted together with metaphase chromosomes could be phosphorylated by recombinant Aurora B kinase *in vitro*. Because topoisomerase II is located along the axis of the chromosomes (Earnshaw and Heck, 1985), while Aurora B is centromeric, topoisomerase II could be phosphorylated only at centromere locations by Aurora B. Considering the dynamic localization of Topoisomerase II during mitosis (Christensen et al., 2002; Tavormina et al., 2002), Aurora B may be able to phosphorylate a significant amount of topoisomerase II through the dynamic pool.

Topoisomerase II has been shown to be essential for chromosome condensation and separation in fission yeast (Uemura et al., 1987). Similar defect of condensation and separation was also achieved by addition of topoisomerase II inhibitors to cultured epithelial cells (Gorbsky, 1994). Although topoisomerase II is required for mitotic chromosome assembly *in vitro*, it is freely extractable after chromosome condensation without altering chromosome structure (Hirano and Mitchison, 1993). These results suggest that topoisomerase II might be required for the initial stage of condensation.

Coincidently, Aurora B has been shown to regulate chromosome condensation and segregation (Adams et al., 2001c; Giet and Glover, 2001). It is likely that Aurora B can phosphorylate topoisomerase II at the centromere and/or chromosomes to execute this function. Knock-out cells of topoisomerase II in *Drosophila* does not affect localization of chromosomal passenger complex to the centromeres and the central spindle (Chang et al., 2003), suggesting that topoisomerase II may not be the docking protein for Aurora kinases at centromeres. However, the possibility that Aurora kinase could regulate topoisomerase II function at centromeres dynamically cannot be ruled out.

Topoisomerase B is the homolog of topoisomerase II in *Dictyostelium*. Co-purification of topoisomerase B with DdAurora in *Dictyostelium* suggests that topoisomerase II could be a substrate/binding partner of Aurora kinase. The localization and function of Topoisomerase B has not been characterized in *Dictyostelium*. It will be intriguing to determine whether it can localize to chromosomes or centromeres during mitosis and whether it is required for the localization of DdAurora to the centromeres. Future research on this potential binding partner of DdAurora may help shed some light on the role of Aurora kinase in early mitosis.

4.3.2 Hsp60 and DdAurora

Hsp60 is a chaperone protein that is essential for the folding and intracellular trafficking of many proteins. It has an essential role in maintaining mitochondrial biogenesis and energetics (Deocaris et al., 2006). Whether it has any relationship to mitosis or cytokinesis has not been reported. Interestingly, expression levels of Hsp60 were found to peak at the G_2/M transition (Martinez-Diez et al., 2006), which is

consistent with Aurora kinase expression and activation. Beyond mitochondrial localization, Hsp60 was also found at the plasma membrane (Soltys and Gupta, 1996; Soltys and Gupta, 1997; Soltys and Gupta, 2000). Membrane-associated Hsp60 forms a complex with histone 2B and is regulated via phosphorylation by type I protein kinase (Khan et al., 1998). Hsp60 may also have a role in peptide transportation (Jones et al., 1994). These results suggest that Hsp60 could be involved in mitosis and might be phosphorylated by Aurora kinase at a novel location, such as cortex region of the cleavage furrow, to regulate transportation of certain peptide to the plasma membrane.

The *Dictyostelium* Hsp60 named HspA has been identified as a mitochondrial chaperonin (Kotsifas et al., 2002). Although HspA is a member of heat shock gene family, the protein is not stress-inducible in response to heat. Since the authors were unable to generate an HcpA null cell line, it is possible that HspA can interact with DdAurora and this interaction is essential for mitosis in *Dictyostelium*. On the other hand, it is possible that HspA works as a chaperone of nascent DdAurora but not a real binding partner, or that DdAurora is somehow involved in mitochondrial biogenesis through the interaction with HspA. Further characterization of HspA function and subcellular localization will help to answer these questions.

Chapter 5 Summary and Future Directions

5.1 SUMMARY

As one of the most important mitosis regulators, Aurora kinases control multiple critical stages of mitosis and cytokinesis, including centrosome maturation and separation, chromosome condensation and segregation, cleavage furrow formation and contraction. In *Dictyostelium*, an excellent model system for studying mitosis and cytokinesis, function of this important kinase had not been previously explored. In this study, I completed a comprehensive functional analysis of DdAurora – the single Aurora kinase in *Dictyostelium*.

Although *Dictyostelium* has only one Aurora kinase, DdAurora shares characteristics of both Aurora A and Aurora B. It has a potential truncated A-box, which is only found in vertebrate Aurora A kinases, as well as the signature Aurora motif in the activation loop and the D-box near the C-terminus (Chapter 2.2.1). The subcellular localization of DdAurora also represents characteristic of both Aurora A and Aurora B kinases. DdAurora localizes to spindle poles and centromeres in metaphase and then a portion of the protein localizes to the central spindle at the metaphase/anaphase transition (Chapter 2.2.3, 2.2.4). During late cytokinesis, DdAurora also localizes to the cleavage furrow (Chapter 2.2.2). Failure to generate a DdAurora null cell line indicates that DdAurora may be essential for *Dictyostelium*. Overexpression of a kinase inactive form of DdAurora kinase abolishes the initiation of cytokinesis (Chapter 2.2.5), suggesting that DdAurora kinase activity may be important for cytokinesis.

A property unique to Aurora B is the ability to interact with the protein INCENP. Similarly, DdAurora forms a chromosomal passenger complex with DdINCENP (Chapter 3.2.2). The conserved C-terminal IN-box domain of DdINCENP is essential for the interaction with DdAurora and also for the central spindle localization of DdAurora (Chapter 3.2.3). The requirement for DdINCENP for the displacement of HcpA from the centromere at the metaphase/anaphase transition (Chapter 3.2.1) is suggestive that DdINCENP is also required for the activation of DdAurora at the centromere. Localization of DdAurora to the central spindle at the metaphase/anaphase transition requires Kif12 (Chapter 3.2.4), a homolog of mitotic kinesin like protein 2 (MKLP2). However, this requirement for Kif12 can be suppressed by overexpression of GFP-DdINCENP (Chapter 3.2.5). In addition, Myosin II heavy chain is required for the proper localization of both DdAurora and DdINCENP to the cleavage furrow during cytokinesis (Chen et al., 2006).

Finally, by performing large-scale immunoprecipitation of wild-type cell extracts, several potential binding partners/substrates of DdAurora have been identified, including topoisomerase B and HspA (Chapter 4.2). Future research on these proteins may help to elucidate DdAurora function in different stages of M phase.

5.2 FUTURE DIRECTIONS

5.2.1 Cleavage Furrow Localization of DdAurora at Very Late Stage of Cytokinesis

By time-lapse fluorescence microscopy of wild-type cells expressing GFP-Aurora I demonstrated that GFP-DdAurora localizes to the cleavage furrow at a very late stage of

cytokinesis. Although *Dictyostelium* do not have a midbody structure in cytokinesis, this late furrow localization of DdAurora greatly resemble the midbody structure in more complex eukaryotes. DdINCENP is also found at the cleavage furrow from telophase to the end of cytokinesis (Chen et al., 2006). It is possible that *Dictyostelium* cells use this late furrow localization of DdAurora/DdINCENP complex in a manner similar to the function of the midbody to finish the final abscission stage of cytokinesis.

In Myosin II heavy chain null cells, this late furrow localization of the DdAurora/DdINCENP complex is disrupted (Chen et al., 2006). Since DdAurora cannot translocate to the central spindle in anaphase in DdINCENP and Kif12 null cells, DdAurora is also missing from the cleavage furrow of these cells (Chapter 3.2.1, 3.2.4). Because the N-terminal domain of DdINCENP is essential for its furrow localization but not for the interaction with DdAurora, it will be interesting to see how introduction of different truncation mutants of DdINCENP can affect the late furrow localization of DdAurora in these null cell lines. Since live-cell microscopy is the best method to observe cells undergoing cytokinesis, fusing DdAurora with another fluorescence tag and co-transforming this construct with GFP-tagged DdINCENP truncation mutants into DdINCENP or Kif12 null cells will help us to examine the late furrow localization of DdAurora in different DdINCENP truncation expressing cell lines.

5.2.2 DdAurora and TACC

Although *Dictyostelium* does not have a homolog of TPX2, the essential binding partner/activator of Aurora A kinase, it has a homolog of another important Aurora A partner – the TACC protein (Koch et al., 2006). It has been shown that Aurora A kinase

is required to localize TACC to centrosomes and TACC is a binding partner/substrate of Aurora A (Giet et al., 2002).

In *Dictyostelium*, it has been shown that TACC colocalizes with DdCP224 at the centrosome in interphase (Koch et al., 2006). By immunostaining wild-type cells expressing TACC-GFP with anti-DdAurora antibodies, I found recently that TACC-GFP colocalized with DdAurora differently from the colocalization of DdCP224 with DdAurora. As shown in Figure 5.1, polar DdAurora localized distally to DdCP224 in metaphase. In contrast, DdAurora colocalized extensively with TACC-GFP at the spindle poles in metaphase. These results suggest that TACC may not exactly colocalize with DdCP224 at the centrosome during mitosis. TACC may interact with DdAurora at the outer side of the spindle pole. Further examination of the interaction between TACC and DdAurora by co-immunoprecipitation will help to elucidate their possible interaction during mitosis.

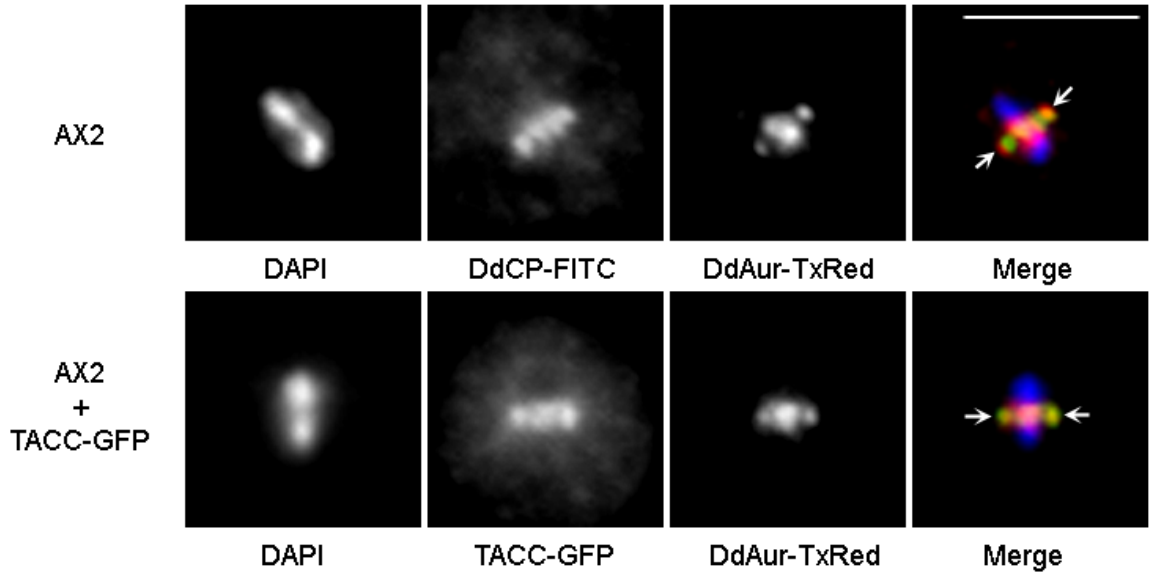

Figure 5.1 DdCP224 and TACC-GFP colocalize with DdAurora differently in metaphase.

In the merged images, DNA is shown in blue, DdAurora in red and DdCP224 or TACC-GFP in green. The top panel shows the colocalization of endogenous DdCP224 and DdAurora in wild-type AX2 cells. In the merged image, DdAurora localized distal to the localization of DdCP224 (shown by small arrows). The lower panel shows the colocalization of TACC-GFP and endogenous DdAurora. In the merged image, TACC-GFP colocalized extensively with DdAurora at the spindle poles (shown by small arrows). Bar, 5μm.

5.2.3 Characterization of TopB and HspA

Topoisomerase B and HspA have been identified as potential binding partners or substrates of DdAurora. Topoisomerase B has not been characterized in *Dictyostelium*. It is predicted to be located in the nucleus (dictybase.org). HspA has been shown as a mitochondrial chaperonin (Kotsifas et al., 2002). No connection to mitosis or cytokinesis has been established for both of them. Close examination of their subcellular distribution during mitosis may provide a clue to their potential role in mitosis regulation. Their interaction with DdAurora should also be confirmed by some other analysis methods.

5.2.4 More Potential Binding Partners

Although I have shown that DdAurora might form a complex with DdINCENP at metaphase centromeres, anaphase central spindle and late cleavage furrow, and might interact with TACC at the spindle poles in metaphase, it is still unclear how DdAurora can keep associated with centromeres throughout mitosis. Further protein purification work may help to address this question. Since expression level and activity of Aurora kinase is highly elevated during mitosis, it will be a good idea to perform large-scale immunoprecipitation on synchronized *Dictyostelium* cell cultures. The other practical method is to perform purification on the kinase inactive form of DdAurora, the inability to phosphorylate the substrate may help to maintain their interaction at a high level.

Chapter 6 Experimental Procedures

6.1 MATERIALS AND METHODS

6.1.1 Cell Culture and Transformation of Constructs

Dictyostelium discoideum AX2 (wild-type) cells were grown in axenic HL5 medium supplemented with penicillin and streptomycin at 19°C. Null cells were grown with additional 5μg/ml blasticidin S hydrochloride. Cells carrying pTX-based plasmids were grown in medium with additional 10μg/ml G418. For stationary culture, cells were grown in plastic Petri dishes. Cells in suspension culture were grown in conical flasks on a rotating shaker at 200rpm. The constructs were introduced into *Dictyostelium* cells by electroporation and the transformants were selected in the HL-5 medium with 10μg/ml G418.

The TACC-GFP construct and monoclonal DdCP224 antibody are generous gifts from Dr. Graf (Graf et al., 2000; Koch et al., 2006). Kif12 null cell line is a gift from Dr. Spudich (Lakshmikanth et al., 2004). The HcpA-GFP construct is a gift from Dr. Nellen (Kaller et al., 2006).

6.1.2 Cloning of DdAurora and Construction of GFP-DdAurora

A 1.6-kb sequence corresponding to the full length of DdAurora gene was cloned from *Dictyostelium* genomic DNA by Polymerase Chain Reaction (PCR) using the 5' and 3' primers AO-499 (5’-CGAGCTCATGAGTTATCCAAATAATAAAGAAAATAGTAACAATATTGGTG-3’)

and AO-500 (5'-CGGATCCTTAATAAGTCATTTGAGATGGTAATGGAAGACCC-3'), respectively. The full-length *DdAurora* gene was further cloned into a pTXGFP vector and a pTAPGFP vector respectively to generate the GFP-DdAurora and TAPGFP-DdAurora constructs. pTXGFP is a generous gift from Dr. Tom Egelhoff (Levi et al., 2000).

6.1.3 Generation of Kinase Inactive Form of DdAurora

Kinase defective form of DdAurora – DdAurora (K139R) was generated with QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the protocol. Primers AO-555 (5'-GAGTCAATTCATTGTTGCACTCAGAGTTTTATTTAAAAATCAATTACAAACAC 3') and AO-556 (5'-GTGTTTGTAATTGATTTTTAAATAAAACTCTGAGTGCAACAATGAATTGACTC -3') were used for the PCR reaction.

6.1.4 Fluorescence Microscopy of Live Cells

For live microscopy of mitotic cells, cells in active log phase growth were first resuspended to a concentration of $2X10^6$ cells/ml. 0.5ml of cell suspension was plated on a small Petri dish with a cover slip mounted at the bottom (MatTek, Ashland, MA). After cells attached to the cover slip, HL-5 medium was removed and replaced with low-fluorescence (LF) medium for at least 20 minutes before observation (Bretschneider et al., 2002). The live imaging of the cells was conducted by using a Nikon Eclipse TE200

microscope (Nikon Instruments, Dallas, TX) equipped with a 100X 1.4 NA PlanFluor Objective, shuttered illumination, and a Quantix 57 camera (Roper Scientific, Tucson, AZ) controlled by Metamorph (Universal Imaging Corp., West Chester, PA). The exposure time for the GFP fluorescence was 100 ms or less with the interval time being at least 10 seconds.

6.1.5 Immunostaining and Microscopy of Mitotic Cells

For immunostaining of mitotic cells, cells in active log phase growth were harvested from stationary culture and resuspended to a concentration of $2X10^6$ cells/ml. 200ul of cell suspension was put on each cover slip and sit still for at least 20 minutes. For fixation, cells were first fixed in 1X PDF buffer (20mM KCl, 11mM K_2HPO_4, 13.2 mM KH_2PO_4, 1mM $CaCl_2$ and 2.5mM $MgSO_4$, pH 6.4) with 2% formaldehyde and 0.01% Triton X-100 for 15 minutes at room temperature, then in dehydrated methanol with 1% formaldehyde at –20°C for 5 minutes. The primary antibody was affinity-purified anti-DdAurora or monoclonal anti-DdCP224. The second antibody was Texas-red conjugated goat-anti-rabbit or FITC conjugated goat-anti-mouse antibody (Molecular Probes, Eugene, OR). For DAPI staining of DNA, cover slips were immerged in 1XPDF buffer with 0.1μg/ml DAPI for 10 minutes before the final wash step.

6.1.6 Antibody Generation and Affinity Purification

A full-length cDNA of DdAurora was cloned from *Dictyostelium* cDNA library (Stratagene, La Jolla, CA) by using the 5' and 3' primers AO-554 (5'-CGAATTCATGAGTTATCCAAATAATAAAGAAAATAGTAACAATATTGGTG-3')

and AO-500 (5'-CGGATCCTTAATAAGTCATTTGAGATGGTAATGGAAGACCC-3'), respectively. The PCR product was further cloned into the pMal-c2X vector (New England Biolabs, Ipswich, MA). MBP-DdAurora fusion protein was expressed in *Escherichia coli* and purified according to the provided protocol. 5ml of Amylose Resin (New England Biolabs, Ipswich, MA) was used for one liter of *Escherichia coli* culture. The purified fusion protein was injected into rabbits to raise polyclonal anti-DdAurora antibodies (Cocalico Biologicals, Reamstown, PA). To purify polyclonal anti-DdAurora antibodies by affinity binding, purified MBP-DdAurora was further cross-linked to AminoLink Coupling Gel (Pierce, Rockford, IL) according to the provided protocol. TAP-tagged GFP protein was purified from *Dictyostelium* cell culture and used to generate polyclonal anti-GFP antibodies by the same procedure.

6.1.7 Affinity Purification of Polyclonal Anti-DdAurora Antibodies

1ml of AminoLink Coupling Gel (Pierce, Rockford, IL) cross-linked with more than 10mg of MBP-DdAurora was used to purify polyclonal anti-DdAurora antibodies from 2ml of crude serum. The crude serum was first pre-cleaned by centrifugation at 13,000 rpm in a desktop centrifuge for 10 minutes. The supernatant was diluted to 10ml with 0.1M sodium phosphate buffer (pH 7.4) and incubated with pre-washed MBP-DdAurora gel for 2 hours at 4°C in a 15ml Poly-Prep Chromatography Column (Bio-Rad, Hercules, CA). The antibody-gel mixture was then packed by gravity in the column and washed extensively with 0.1M sodium phosphate buffer. Bound anti-DdAurora antibodies were finally eluted by 100mM glycine (pH 2.5) and equilibrated to pH 7.4

immediately with 1M Tris-HCl (pH 8.0). Purified anti-DdAurora antibodies were kept in 4 °C ice water bath afterwards.

6.1.8 TAP-tag Purification

pTAPGFP vector was constructed by Joe Mireles in our laboratory. A coding sequence of TAP-tag was inserted to the 5-prime of GFP cassette. The procedure for TAP-tag purification was modified from the one described by Rigaut (Rigaut et al., 1999). For large-scale purification, 2 liters of *Dictyostelium* cells expressing TAP-GFP were cultured in a 6-liter flask shaking at 180rpm. Cells were harvested when the density reached ~5X10^{6}cells/ml. Cells were collected at 3000rpm for 10 minutes in a Sorvall RC-3B centrifuge (Global Medical Instrumentation, Inc, Ramsey, MN) and washed once with 1XPDF buffer. The pellet was then resuspended in 100ml of lysis buffer (50mM Tris-HCl, 50mM KCl, 2mM $MgCl_2$, 0.5mM DTT, 100mM NaCl, pH8.0) with protease inhibitor cocktail (1:100, Sigma Chemical Co., St. Louis, MO). The cell suspension was lysed by sonication for 8x1 minute at 1 minute interval. To ensure the complete lysis, 10ul of the lysate was examined on a hemocytometer with a light microscope. The cell lysate was centrifuged at 14,000 rpm for 30 minutes to clean up the pellet. The supernatant was mixed with 200ul pre-washed IgG agarose beads (Amersham Biosciences, Uppsala, Sweden) and incubated on a rocking plate for 2 hours at 4°C.

After incubation, the beads suspension was packed into a 15ml Poly-Prep Chromatography Column (Bio-Rad, Hercules, CA) by gravity and washed with 30ml IPP150 buffer (10mM Tris-HCl, 150mM NaCl, 0.1% NP40, pH 8.0) followed by 10ml of TEV cleavage buffer (10mM Tris-HCl, 150mM NaCl, 0.1% NP40, 0.5mM EDTA, 1mM

DTT, pH 8.0). If this purification step is performed successfully, a light green color, which is the color of the TAP-GFP recombinant protein, should be observed from the IgG beads. Then, 100 units of TEV protease (Invitrogen, Carlsbad, CA) were added with 1ml of TEV cleavage buffer to the column. The suspension was incubated at 18°C for 2 hours and then at 4°C overnight on a rotating rack. The suspension could be transferred into an eppendorf tube to achieve better agitation.

The next day, the digestion suspension was transferred back to the column and the flow through was collected in a fresh tube. If the digestion is efficient, you should be able to see light green color from the flow through. Rinse the beads with another 2X0.5ml of TEV cleavage buffer and put the flow through together with the previous one. 6ml of calmodulin binding buffer (10mM Tris-HCl, 150mM NaCl, 0.1% NP40, 10mM β-mercaptoethanol, 1mM MgAc, 1mM imidazole, 2mM $CaCl_2$, pH 8.0) and 6µl $CaCl_2$ were added to the elute in another 15ml column. 200µl of pre-washed calmodulin beads (Stratagene, La Jolla, CA) were also added. The suspension was incubated for 1 hour at 4°C to allow the binding of tagged protein to the beads. Finally, after washing the beads with 30ml calmodulin binding buffer, the bound protein was eluted by 5X0.5ml elution buffer (10mM Tris-HCl, 150mM NaCl, 0.1% NP40, 10mM β-mercaptoethanol, 1mM MgAc, 1mM imidazole, 2mM EGTA, pH 8.0).

6.1.9 Co-immunoprecipitation with DdAurora Antibodies

Affinity purified polyclonal anti-DdAurora antibodies were used in all the immunoprecipitation experiments. Cells expressing target GFP-fusion protein were

cultured in suspension in a 250ml flask. When the concentration reached ~2X10^6 cells/ml, actively dividing cells are harvested by centrifugation at 1,500 rpm for 5 minutes. They were washed in 1xPDF buffer once and then resuspended in 3ml of 0.1M sodium phosphate buffer (pH 7.4) with fresh addition of 1:100 protease inhibitor cocktail and 1mM DTT. Cells were lysed by sonication for 6x15 seconds with 15-second rest in between. The lysate was then centrifuged at 13,000 rpm for 15 minutes at 4°C twice to clean up cell debris. 40µl of purified anti-DdAurora antibodies were added to 1ml of supernatant after centrifugation. Either unspecific Rabbit IgG or no addition of antibody was used as a negative control. The mixture was rotated at 4°C for 1 hour and then 20µl of pre-washed protein A beads (Amersham Pharmacia, Piscataway, NJ) were added to the suspension. After another 30 minutes incubation, the antibody-protein A complex was spun down at 3,000 rpm for 1 minute and washed by 6x1ml 0.1M sodium phosphate buffer. Finally, bound proteins were eluted by boiling the protein-bead complex in 100µl RSB loading buffer at 95°C for 10 minutes. The elutions were resolved on a 10% poly-acrylamide gel and examined by western blot analysis with anti-GFP antibodies.

For large-scale immunoprecipitation, same procedure was used with 10 times more material and reagent, except in the final elution step, only 200µl of RSB was used to boil proteins off from the beads.

6.1.10 *In Vitro* Pull-down with MBP-DdAurora

Because different DdINCEP truncations have very different expression level in DdINCENP null cells, *in vitro* assay was used instead of co-IP to keep DdAurora input

amount at the same level. Cells were cultured and harvested in the same way as that of co-immunoprecipitation experiment. After sonication, GFP-DdINCENP-ΔC lysate was diluted 10 times before centrifugation. By this dilution, the input levels of GFP-fusion proteins were adjusted to the same level too. After centrifugation, 200µl of MBP-DdAurora saturated amylose beads were added to the supernatant and incubated at 4°C for 2 hours. After extensive wash, the bound proteins were boiled off from the beads by 100µl RSB. The elutions were also resolved on a 10% poly-acrylamide gel and examined by western blot analysis with anti-GFP antibodies.

6.1.11 Immunoblot Analysis

Samples were separated on 10% acrylamide mini-gels (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membranes, and blocked with 5% dry milk in 1X TBS buffer. Membranes were incubated in polyclonal anti-GFP primary antibodies (1:1000 dilution), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Southern Biotech, Birmingham, Alabama; 1:5000 dilution). Bound antibodies were detected using a SuperSignal West Pico Chemiluminescent kit (Pierce, Rockford, IL).

6.2 PLASMIDS AND CELL LINES

Table 5.1 Plasmids and Cell Lines Used in this Study

Plasmids	Description
pTX-DdAurora	Full-length DdAurora cloned in GFP-tag vector for examination of subcellular localization of DdAurora. N-terminal GFP tag. G418 resistance.
pTX-DdAurora(K139R)	Expression construct for expressing kinase inactive form of GFP-DdAurora. The ATP binding site Lysine139 was replaced by Arginine (Terada et al., 1998). N-terminal GFP tag. G418 resistance.
pTAPGFP-DdAurora	Full-length DdAurora cloned in TAPGFP-tag vector for protein expression and purification in *Dictyostelium*. N-terminal TAPGFP tag. G418 resistance.
pMal-c2X-DdAurora	Full-length DdAurora cDNA cloned in bacterial expression vector for protein purification and polyclonal antibody production. Ampicillin resistance.
pTX-DdINCENP (by Qian Chen)	Full-length DdINCENP cloned in GFP-tag vector for examination of subcellular localization of DdINCENP (Chen et al., 2006). N-terminal GFP tag. G418 resistance.
pTX-DdINCENP-ΔN (by Qian Chen)	The last 833 amino acids of DdINCENP, including the IN-box, cloned in GFP-tag vector for examination of subcellular localization of DdINCENP-ΔN (Chen et al., 2007). N-terminal GFP tag. G418 resistance.

pTX-DdINCENP-ΔC (by Qian Chen)	The first 1013 amino acids of DdINCENP, not including the IN-box domain, cloned in GFP-tag vector for examination of subcellular localization of DdINCENP-ΔC (Chen et al., 2007). N-terminal GFP tag. G418 resistance.
pFL-TACC-GFP (by Katrin V. Koch)	Full-length TACC cloned in GFP-tag vector for examination of subcellular localization of TACC (Koch et al., 2006). C-terminal GFP tag. BSR resistance.
pHcpA-GFP (by Markus Kaller)	Full-length HcpA cloned in GFP-tag vector for examination of subcellular localization of HcpA (Kaller et al., 2006). C-terminal GFP tag. G418 resistance.
pGFP-Kif12 (by Gandikota S. Lakshmikanth)	Full-length Kif12 cloned in GFP-tag vector for examination of subcellular localization of Kif12 (Lakshmikanth et al., 2004). N-terminal GFP tag. G418 resistance.

Cell lines	Description
AX2	Wild type axenic strain; grows in HL-5 media supplemented with 0.6% penicillin/streptomycin (P/S)
DdINCENP null (10B6) (by Qian Chen)	Derived from AX2 parent strain, *icpA* gene disrupted by blasticidin cassette (Chen et al., 2006); grows in HL-5 media supplemented with 5μg/ml blasticidin.
Myosin heavy chain null (by Arturo De Lozanne)	Derived from AX2 parent strain, *mhcA* gene disrupted by G418 cassette (De Lozanne and Spudich, 1987); grows in HL-5 media supplemented with 10μg/ml G418.
Kif12 null (by Gandikota S. Lakshmikanth)	Derived from AX2 parent strain, *kif12* gene disrupted by blasticidin cassette (Lakshmikanth et al., 2004); grows in HL-5 media supplemented with 5μg/ml blasticidin.

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